Shoot regeneration and *Agrobacterium*-mediated transformation of carnation

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Shoot regeneration and *Agrobacterium*-mediated transformation of carnation

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CHAPTER 1

GENERAL INTRODUCTION

A.C. van Altvorst

1 Introduction

The work described in this thesis comprises the development of a regeneration and transformation system for carnation (*Dianthus caryophyllus* L.). The project was supported financially by the Dutch Ministry of Economic Affairs and five Dutch carnation breeding companies, viz Hilverda B.V., M. Lek en zonen B.V., P. Kooij en zonen B.V., Van Staaveren B.V. and West-Select B.V. The companies supported the project, because they would like to make carnation accessible to genetic modification. As an introduction to this thesis, a general review is given including a description of the existing carnation types, some yield and sales statistics of various carnation types, and the position of the Dutch carnation in the international market of today. Subsequently, the different breeding topics are discussed concerning the prospects of genetic engineering. These results were based on interviews with the Dutch carnation breeding companies mentioned above.

2 Carnations and their importance

Carnation types

The carnation originates from the coastal regions of the Mediterranean. The botanical (wild) carnation is seasonal and it flowers in spring and summer. The flowers have a strong scent and a fast senescence. After many years of crossing and selection these characteristics have virtually disappeared. Various types have been developed as a result of crossing (Figure 1, colour plate 1). Commercial cultivars are vegetatively propagated and can be divided into two main groups, the standard and spray carnations (for a review, see Hughes 1993). A minor group is formed by the pot carnations.

Standard carnations

Standard carnations are produced by disbudding, removal of all lateral flower buds and leaving the terminal flower. Standard carnations have to be disbudded continuously, which is essential to maintain flower size and quality. The oldest carnation type is the standard carnation with a long, heavy stalk. During the early 1800s, the development of carnation cultivars started in Southern France and Northern Italy. In 1852, the first carnation seedlings were imported into the

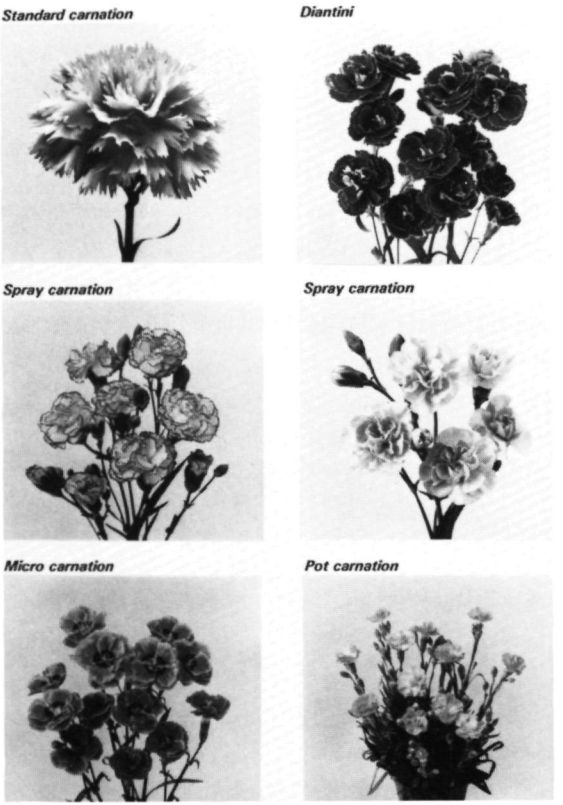

Figure 1. Various carnation types as a result of crossing.

U.S.A., and since then, carnation breeding was also carried out in U.S.A. 'William Sim' is an American cultivar obtained by W. Sim in 1938. 'William Sim' and its mutants were very successful, resulting in an expansion of breeding activities (reviewed by Holley and Baker, 1963). Sim cultivars are old, but the flower production is reasonable. Nearly all colours were available and in absence of an alternative, they dominated the market for a long time. Its tendency to split flowers and its great sensitivity to the soil fungus *Fusarium oxysporum* has led to the 'Mediterranean type', developed in France and Italy. At the moment, standard carnation cultivars are mostly crosses between the Sim and the Mediterranean type. Since 1980, some other standard carnations have appeared on the market: the midi carnation, 25 % smaller than the original standard

carnation, and the mignon carnation, also a small type of standard carnation, but in this type the main flower and side-buds are left. Midi and mignon carnations only represent a small percentage of the total carnation production. Carnation breeding activities have been reviewed by Holley and Baker (1963, 1991).

Spray carnations

Unlike in standard carnation, in spray carnations the terminal flower bud is removed and the lateral buds are retained, which results in a more open type. In 1956, the spray carnation was developed in the U.S.A. by Thompson and it was introduced in Europe in 1964 (for a review, Holley and Baker, 1963, 1991). From 1980 onwards, also other carnations have been marketed; the micro carnation, a small type of spray carnation in which all the flower buds are retained, and the Diantini carnation, a tetraploid type in which the terminal bud is removed and the lateral buds are retained. Both types still form a small percentage of the total carnation supply.

Sales in the Netherlands

Worldwide, carnation is cultivated on about 10.000 hectares; it is the most important floricultural crop. In the Netherlands, according to statistical data of 1992 of the Produktschap voor Siergewassen, the total area of carnations in greenhouses was 225 ha, from which 76 ha of standard carnations and 149 ha of spray carnations. The acreage of open field cultivation was estimated at 50 to 60 ha, mainly used for spray carnations. About 500 Dutch companies are growing carnations in greenhouses, and another 160 companies cultivate carnations outdoors. In 1992, the total carnation sales sold via the Dutch auctions (both imported and Dutch carnations) accumulated to 278 million Dutch guilders. It ranked at the third position of ornamental crop sales next to rose (with sales of 833 million) and chrysanthemum (562 million).

The costs for carnation cultivation in Dutch greenhouses are relatively high. African and South American countries have a competitive advantage due to low labour costs and low investments due to an optimal climate. At the moment, Kenya, Colombia, Israel, Spain, Japan and Italy are the most important competitors. The world production centers for today's commercial carnations are reviewed by Whealy (1992). The import of carnation into the Netherlands is still increasing, thus Dutch breeders have to improve the quality of the cultivars and growers have to cultivate an exclusive quality. Breeding companies are

trying to set up a reference assay to classify the quality of carnations. Such a quality classification can result in clear differences in prices, and it may stimulate the growers to produce carnations with a higher quality. At the moment, carnation growers have the following difficulties: yields are low, the Dutch government has restricted the use of chemicals, future investments are needed to comply with environmental rules, there is yet no quality distinguishing feature, prices are low and growers are faced with a fierce competition. In the past ten years the diversification in cut flower production has led to a relative weak position of the carnation within the assortment. This is partly compensated by diversification within the carnation assortment.

3 Biotechnology in carnation breeding; a breeder's perspective

Introduction

Plant biotechnology includes the area of research that combines tissue culture and molecular techniques with the aim of obtaining plants with improved agronomical and value added traits. In conventional breeding the introduction of new traits is achieved by crossing, a random process in which the paternal and maternal genomes are mixed and the desired phenotype has to be selected among thousands of individuals in a highly heterozygous progeny. Using transformation techniques, the recipient plant retains its genetic information and only gains the added traits. Moreover, genetic engineering offers the opportunity to introduce all kinds of genes, bypassing existing crossing barriers. This makes it possible to introduce genes from outside the plant kingdom. In fact, most of the genes so far introduced into plants, originate from bacteria and viruses, because these are more accessible to molecular genetics. Molecular techniques are being developed enabling the isolation of plant genes. It is expected that in the near future many genes will be available for the improvement of quality and disease resistance in plants. In this section, the use of biotechnology in carnation breeding will be discussed. The results were based on interviews with researchers or managers of five Dutch breeding companies. The study was aimed at answering the following questions:

1) What are the goals for current carnation breeding?
2) What are the reasons for financing research on regeneration and transformation at joint expense?

3) Which of the existing carnation cultivation problems would the breeders like to be solved by genetic engineering?
4) Do the breeders envisage that genetic engineering will be profitable?
5) What is their opinion concerning the acceptance of transgenic products by the consumers?

The study was set up in two parts. First there was an open oral interview with researchers of each of the five companies, followed by an interview using a questionnaire. The extensive discussions in the first part were set up to gain insight into the most relevant aspects. This resulted in a general view concerning breeding topics and attitudes towards genetic engineering. The second study was carried out to classify the most important breeding topics and biotechnological applications. The breeders were asked to rank the breeding topics in sequence of importance.

Breeding topics

According to the interviewed breeders, carnation breeding in their company is focused on topics mainly determined by growers, salesmen and consumers. The demand of consumers is largely determined by the type of flower and colour. Due to the rise in sales of bouquets, product diversification becomes more and more important. Salesmen prefer carnations which can easily be stored and transported. For growers, yield and handling are also very important.

Like most crops, the culture of carnation is threatened by a wide range of diseases and pests (reviewed by Holley and Baker, 1991). To cope with this problem growers are forced to use copious amounts of chemical controlling agents. In 1992, the use of chemical nematicides, fungicides and insecticides in the Dutch carnation culture was estimated at 31.4 kg per ha (Source: Centraal Bureau voor de Statistiek). The extensive use of pesticides and insecticides is no longer acceptable, because of environmental pollution and the dangers for human and animal health. As indicated in the National Environmental Policy Plan (Ministerie van V.R.O.M., 1989), the Dutch government is strongly limiting the use and emission of hazardous chemicals. This increases considerably the need for disease and pest resistant cultivars. In current carnation breeding a long list of breeding objectives can be made. These include: development of new flower forms, increase in flower colour range, resistance to insects and diseases, postharvest longevity, extension of low light and temperature tolerance, increase of winter production, elimination of disbudding, improvement of branching in spray carnations, increase in yield, stem strength, compactness,

reduction of calyx splitting and enhancement of fragrance. Table 1 lists the five breeding topics which were indicated by the breeding companies as most important and include successively (1) resistances against pests and diseases, (1) yield, (3) prolonged vase-life, (4) absence of lateral shoots (in standard carnations) and (5) plant habitus. In the following section, these topics will be described in more detail and the prospects of biotechnological application will be discussed.

Table 1. Rating of classical and biotechnological breeding traits, relative to other items. Results are based on interviews with five Dutch breeding companies. Per breeding company, the breeding topics were numbered in sequence of importance. The most important item received one point, the second two points, and so on.

(a)	Most important breeding topics	Points
1	Resistance to pests and diseases	14
1	Yield	14
3	Prolonged vase-life	20
4	No lateral shoots (in standard carnations)	22
5	Plant habitus	25
(b)	Most important pest and insect diseases in carnation culture	
1	*Fusarium*	7
2	Thrips	13
3	*Alternaria*	19
4	Viruses	21
5	*Botrytis*	28
(c)	Traits which need to be improved first by genetic engineering	
1	Resistance against *Fusarium*	11
2	Prolonged vase-life	17
3	Yield	21
3	Resistance against thrips	21
5	Resistance against viruses	22
(d)	Traits which will be applied first by genetic engineering in practice	
1	Prolonged vase-life	5
2	Resistance against *Fusarium*	12
3	Blue colour	15
4	Resistance against thrips	26
5	Resistance against viruses	29

Resistances to pests and diseases

Carnations suffer greatly from a wide range of diseases and pests. The five most important pathogens, according to the interviewed breeders, are classified in Table 1. They include successively *Fusarium*, thrips, *Alternaria,* viruses and *Botrytis*. For each pathogen, a short description of current strategies to obtain resistance is given.

Fusarium, Alternaria, Botrytis - Until now, the emphasis of breeding was mainly focussed on *Fusarium* resistance (reviewed in Trujillo *et al.*, 1989). *Fusarium oxysporum f. dianthi* is regarded as the most devastating fungal pathogen affecting carnations. *Fusarium* enters the plant through the roots, develops in the vascular tissue and plants become distorted due to wilting (Baayen and Elgersma, 1985). *Fusarium* is a big problem, due to the costs of soil disinfection. A reasonable level of *Fusarium* resistance has been achieved, due to many years of breeding. Other frequently occurring fungal diseases are *Alternaria blight* and *Botrytis*. *Alternaria blight* causes leaf spot or branch rot and *Botrytis cinererea* causes *Botrytis* flower blight. The blight is common during storage of cut carnations and affects the petals. Breeding for *Alternaria* or *Botrytis* resistance is not yet possible, because reliable reference assays are not available.

Natural pathogen resistance in plants is, in many cases, related to a hypersensitive response (HR) and correlated with the production of PR-proteins. The HR, characterized by a quick, localized necrosis (cell-death) at the infection site, is often accompanied by only a limited spread of the pathogen to non-infected parts of the plant, and can therefore be considered as a host-defense reaction. PR-proteins, which are induced by a pathogen infection, have been demonstrated in more then 20 different species (reviewed by Dons *et al.*, 1991). Some PR-proteins show enzymatic activity, such as chitinase and ß-1,3-glucanase activity. The substrates for these enzymes, chitin and ß-1,3-glucan, are important structural compounds of the cell wall of many fungi. Recently, it was shown that specific chitinases and ß-1,3-glucanases exhibited inhibitory activity towards *Fusarium solani* in transgenic tomato plants (Van den Elzen *et al.*, 1993). It seems likely that *Fusarium* resistant carnation plants might be obtained in a similar way by genetic modification.

Thrips - Thrips is the most damaging insect pest; it causes streaks on the flowers (Trujillo *et al.*, 1989). A number of thrips species are common in carnation, e.g. *Frankliniella occidentalis* and *Thrips palmi*. Thrips nymphs and adults suck plant sap and cause distortion, streaking, and spotting of flowers, foliage, and stems. Dutch breeding companies would like to select for thrips resistance. However, there is yet no reference assay available that is reliable, quick and inexpensive.

Insects often produce trypsin and/or chymotrypsin in their intestinal tract for food digestion. It is generally believed that proteinase inhibitors play some role

in the plant defense system (reviewed by Gatehouse *et al.*, 1991). By producing these inhibitors plants may protect themselves against insects by damaging the insect digestive system. In order to obtain insect-resistant tobacco plants, Hilder *et al.* (1987) successfully introduced the coding sequence for a trypsin inhibitor from cowpea into tobacco plants. Combination of several proteinase inhibitors and other inhibitors of digestive enzymes may provide effective resistance (reviewed by Gatehouse *et al.*, 1991). Because the problem of thrips (*Frankliniella occidentalis*) is world wide and affects many crops, it will be valuable to obtain suitable genes, like genes coding for proteinase inhibitors, and subsequently introduce these genes by transformation into a variety of crops. In this way, thrips resistant carnation cultivars might be obtained in the future.

Viruses - Viruses can severely affect marketability and profitability by reducing the flower quality and the flower production. The four most common carnation viruses are carnation streak virus (CSV), carnation mosaic virus (CMV), carnation mottle virus (CMoV), and carnation ringspot virus (CRSV). Viruses can be transmitted through vectors, such as the green peach aphid (*Myzus persicae*), which is also a serious insect pest on carnations. Aphids reduce plant vigor and secrete honeydew, which reduces quality. Viruses can also be transmitted through vegetative propagation and contaminated harvesting tools. By using clean cuttings from certified virus-free stock plants, viruses can be controlled, but this is time-consuming and expensive. To obtain virus-resistant transgenic plants, various strategies have been developed (reviewed by Dons *et al.*, 1991), the most successful one is the 'coat protein mediated cross-protection' approach (Abel *et al.*, 1986). This is based on the finding that infection of a plant by a virus suppresses or postpones symptoms caused by super-infection with a second more virulent strain. A DNA copy coding for the viral coat protein has been successfully introduced into plants in several plant-virus combinations. Viruses occurring in carnation have not been studied extensively, but no specific barriers seem to block implementation of this approach in carnation.

Yield and plant habitus

The market is competitive and as breeders strive for increasing their market share, they are continuously releasing new varieties in the hope of capturing that ephemeral attention of the consumer. More and more time is spent on the development of new carnation types, which can be wrapped up easier and

transported cheaper, and which have a quick production, a high yield and low production costs. By genetic engineering, it is possible to introduce completely new features into plants, as long as they are monogenic or oligogenic. Characteristics, which are polygenic, like yield and plant habitus, will not likely be modified by genetic engineering in the near future.

Prolonged vase-life

The phytohormone ethylene plays a critical role in the initiation and regulation of senescence (for a review, see chapter 7 of this thesis). Most of today's commercial carnations are sensitive to ethylene, both endogenously produced or exogenously supplied. Because silver-ions bind to the ethylene receptor, and in this way inhibit ethylene action, a post-harvest treatment with silver thiosulfate (STS) results in a delay of flower senescence. Since pretreatment with the heavy metal containing STS will not remain acceptable for long, breeding for longer vase life becomes more important. For several years, breeders have selected ethylene insensitive cultivars early in their breeding programs, resulting in a higher level of tenability in the present carnation population. A good reference assay for longevity could help to select for carnation cultivars with an improved vase-life. Breeding companies are also quite convinced that the biotechnological approach to prolong the vase-life will be applied for commercial cultivars within a few years. The ethylene biosynthesis pathway has already been fully identified (reviewed in chapter 7). Preliminary experiments with antisense suppression of ACC oxidase in transgenic carnation plants showed inhibition of petal inrolling, increasing the postharvest life of the flowers (Michael *et al.*, 1991). This shows that genetic engineering of ethylene metabolism will soon be applicable in carnation breeding. The results of the interviews showed that most breeders doubt whether this strategy will be more cost effective than classical breeding. But, most breeders believe that it is worthwhile to investigate and therefore, the five Dutch breeding companies have recently decided to finance a project with the aim to develop transgenic carnations with an improved vase-life.

Absence of lateral shoots

Standard carnations without lateral shoots in the upper part of the plant are commercially very interesting, because the labour intensive disbudding would no longer be needed. Because carnation is a crop which needs to be vegetatively propagated, lateral shoots have to be present in the lower part of the plant.

Lack of knowledge about the mechanism of action and difficulties in identifying the genes involved limit progress in this area.

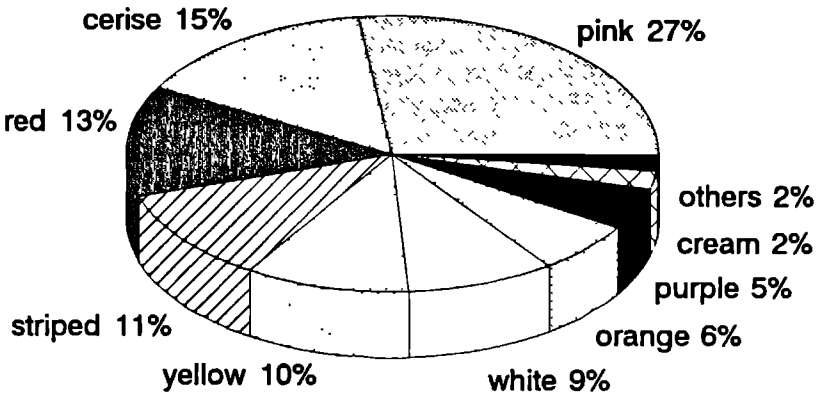

Figure 2. Various flower colours of carnations produced in the Netherlands in 1992. (Source: Produktschap voor Siergewassen).

Flower colour

Because of the large variation within the gene pool, the introduction and selection of new flower colours can easily be improved by classical breeding or by mutation breeding. As flower colour can easily be changed by mutation breeding or cross-breeding, the carnation breeding companies believe that genetic engineering of flower colour might only be profitable for cultivars of outstanding quality. Figure 2 shows the various flower colours for produced carnations in the Netherlands in 1992. In most plant species flavonoids are the most important flower pigments and therefore determine the flower colour (reviewed by Van Tunen and Mol, 1991). In order to obtain a flower with a different set of flavonoids, and therefore an altered colour, several approaches can be followed. First, inhibition of part of the flavonoid pathway leads to accumulation of flavonoid intermediates which might result in a change of flower colour or colour pattern. Two strategies have been developed for the down regulation of gene expression, 'co-suppression' and 'antisense mRNA'. The antisense strategy was based on the prediction that antisense RNA would interfere with the normal sense RNA and thereby cause a suppression of gene expression (Van

der Krol *et al.*, 1988). The co-suppression strategy was based on the finding that transgenes can influence each other's expression and the expression of resident genes in transgenic plants. The molecular mechanisms involved are not yet understood (reviewed by Kooter and Mol, 1993).
Plant species which lack the ability to produce certain flavonoid subgroups can be supplemented with genes encoding the missing enzymes (Van Tunen and Mol, 1991). In order to open a new spectrum of blue and purple pigments, key genes of the delphinidin pathway (such as 3', 5'-hydroxylase) have to be introduced resulting in the production of the blue colour (reviewed by Meyer, 1991). Besides, the vacuolar pH, the presence of co-pigments such as flavonols, and the concentration of anthocyanins also affect the blueness of the flavonoid pigments. The breeding companies believe that introduction of new colours like blue and black will not be profitable. At present, these colours gain a lot of interest because they are not yet available. As soon as blue carnations are achieved and available in the market, they will loose their novelty. Breeders do not expect that blue carnations will give sufficient return in investment.

Possibilities of genetic engineering in carnation breeding
By financing the research project described in this thesis, the Dutch breeding companies directly gain information about the possibilities of carnation transformation. It is not possible to make long-term investments (over ten years), so the research should lead to profitable results in a short time. Part of the research is the development of procedures for the production of adventitious shoots. Such a regeneration protocol will be quickly used in mutation breeding and will be a direct spin off of this research. Besides, the interviews clearly revealed that breeding companies expect good prospects for developing a transformation protocol and its application in carnation breeding.
Genetic engineering is expensive and will therefore only be profitable for the most interesting genes, only for qualities which reduce production cost, such as *Fusarium* resistance and improved vase-life. On the contrary, characteristics, like species foreign colours, are not essential, but desirable. Genes for such traits will only be worth introducing if they become available from other crops and if transformation costs are really low.
According to the breeders, the most important traits that need to be improved by genetic engineering are respectively *Fusarium* resistance, improved vase-life, yield, thrips and virus resistance (Table 1). The application of genetic engineering techniques is hampered by the number of available genes. Basic

knowledge about pathogenesis, screening procedures and inheritance of resistance are important to obtain disease and pest resistance genes. On the other hand, genes isolated from viruses and bacteria have successfully been used for the engineering of resistant plants. The breeding companies believe that application of genetic engineering in carnation will successively result in flowers with an improved vase-life, in *Fusarium* resistant plants, flowers with a blue colour, thrips and virus resistant plants (Table 1). Breeders are convinced of the beneficial possibilities of genetic engineering, but they are less convinced about its widespread applications, as they expect that in most cases genetic engineering will be more expensive than classical breeding. Through royalties, it will take a long time before breeders get a return on investment.
The breeding companies are aware of the problems concerning acceptance of transgenic plants which might hamper a quick use in practice. Introduction of the first transgenic plants will determine the acceptance, so it will be easier if the introduced genes are perceived to be highly beneficial to society. For example, introduction of disease resistance genes that may lower chemical usage.

4 Aim and outline of this thesis

The aim of the research described in this thesis is the development of a regeneration and transformation procedure of carnation. One of the more critical factors in the genetic transformation is the ability to regenerate a mature plant from a single cell. Therefore in this thesis much attention was paid to shoot regeneration of carnation. In chapter two a shoot regeneration procedure is described. Explants from petals and leaves of *in vitro* grown plants were used and adventitious shoot formation was developed at the bases of the explants. The efficiency of adventitious shoot formation from leaf explants of carnation was further improved by changing the preparation of the explants (chapter 3). In chapter four, adventitious shoot formation was studied with leaf, stem and axillary bud explants. To determine which regeneration system would be the most suitable for *A.tumefaciens*-mediated transformation, we have compared the described shoot regeneration procedures.
In many crop species, transgenic plants have been obtained via various gene transfer approaches, including *Agrobacterium*-mediated transformation, direct DNA uptake, microinjection and particle bombardment. The plant pathogenic soil

bacterium *Agrobacterium* is able to transfer foreign genes to the genome of plants and to stably integrate these genes in the plant DNA. This process is efficient in many dicots, however, it is extremely difficult to apply on monocots. As carnation belongs to the dicotyledonous plant species, we chose to develop an *Agrobacterium*-mediated gene transfer protocol. Gene transfer by *A.rhizogenes* was about 20 times more efficient than *A.tumefaciens* transformation. Therefore, *A.rhizogenes* was used to infect carnation petal and leaf explants (chapter 5). The results presented in chapter 5 indicated that regeneration of transformed Ri carnation callus was not succesful. We therefore set out to develop a transformation system with a derivated *A.tumefaciens* strain. In chapter six, successful gene transfer to carnation leaf and petal explants is described, using *A.tumefaciens*. The *A.tumefaciens*-mediated transformation procedure we developed will enable the application of genetic transformation strategies in improving carnation cultivars. In continuation of the transformation project, the five breeding companies have financed a project with the aim to develop transgenic carnations with an improved vase-life. Chapter seven summarizes studies that have provided much insight into the role of ethylene in flower senescence.

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CHAPTER 2

ADVENTITIOUS SHOOT FORMATION FROM *IN VITRO* LEAF EXPLANTS OF CARNATION (*DIANTHUS CARYOPHYLLUS* L.)

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(in Scientia Horticulturae (1992) 51: 223-235)

Summary

A regeneration procedure was developed for carnation (*Dianthus caryophyllus* L.) using leaves from *in vitro* grown plants. Leaves were carefully removed from the stem using forceps. Basal explants were incubated on MS medium with 0.3 mg/l 6-benzyladenine (BA) and 0.3 mg/l α-naphthaleneacetic acid (NAA). After two weeks, adventitious shoots developed at the bases of the explants. The position of leaves on the plant appeared to be important, the youngest leaves just below the apical meristem giving the best regeneration results. The regeneration procedure was generally applicable. Of the various carnation cultivars tested, 24 out of 25 showed regeneration. Using petals as starting material, 37 out of 55 cultivars produced shoots, although due to premature flowering, it was usually impossible to transfer regenerated shoots to soil. Regenerants from leaf explants showed no aberrant plant growth. A number of factors was analyzed to optimize the procedure. Both BA and NAA in the range from 0.1 to 0.9 mg/l affected the average numbers of shoots per regenerating explant, but not the regeneration percentage. The highest number of adventitious shoots was obtained on medium containing 0.9 mg/l BA and 0.3 mg/l NAA. The two leaves of a leaf pair responded differently with the leaf removed secondly from the stem showing the highest regeneration percentage. Combining the optimum conditions, 65% of the explants showed regeneration with an average of 10 shoots per explant.

Introduction

In carnation (*Dianthus caryophyllus* L.), culture systems using meristems have been established for *in vitro* multiplication (Hackett and Anderson, 1967; Weryszko and Hempel, 1979; Roest and Bokelman, 1981; Leshem, 1986) and virus eradication (Baker and Phillips, 1962). Adventitious shoot formation has been described using explants from petals as starting material, but vitrification and premature flowering of the shoots is a severe problem (Kakehi,1979; Gimelli *et al.*,1983; Gimelli *et al.*,1984). Adventitious shoot regeneration has also been obtained using stem explants (Dommergues and Gillot, 1973; Lubomski and Jerzy, 1989), ovules (Demmink *et al.*, 1987), anthers (Villalobos, 1981), macerated shoot tips (Johnson, 1980) and axillary bud explants (Miller

et al., 1991). Although some investigations have focussed on adventitious shoot formation from leaf explants, carnation appeared to be rather recalcitrant in this respect (Roest and Bokelman, 1981; Miller *et al.*, 1991).

Direct regeneration methods, in which adventitious shoots or somatic embryos develop without an intermediate callus phase, may be used for several purposes, such as mutation breeding and *Agrobacterium*-mediated gene transfer. Avoidance of a callus phase reduces the problem of somaclonal variation. As part of a research programme on the development of an *Agrobacterium*-mediated gene transfer system in carnation, this study was aimed at establishing an adventitious shoot regeneration procedure, which was applicable year round and not dependent on variable seasonal conditions of donor plants grown in a greenhouse. A regeneration protocol is described for the production of adventitious shoots from leaf explants from *in vitro* grown plantlets. The protocol was optimized regarding the growth conditions of the donor plants, the isolation of the explants, the hormone concentrations of the medium. It was tested on a large number of cultivars and compared to the regeneration from petal explants.

Materials and methods

Plant material

Fifty-five cultivars were used representing the various carnation types: Mediterranean, Sim, spray, Diantini, micro carnations and some interspecific hybrids. The cultivars are indicated with CPRO clone numbers. Plants, obtained as rooted cuttings from commercial sources, were grown in a greenhouse at a temperature of 16/14°C (day/night temperature). Nodal cuttings were taken to establish an *in vitro* stock plant culture. These were surface sterilized for 15 min in a 2% (v/v) solution of sodium hypochlorite (commercial bleach) with 0.01% (v/v) Tween-20, rinsed three times for 10 min in sterile water and transferred to glass jars with multiplication medium. The multiplication medium contained MS anorganic salts (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose (MS-30), 0.5 g/l trypton, 0.1 mg/l α-naphthaleneacetic acid (NAA) and 0.7% (w/v) purified agar (Oxoid) and was adjusted to pH 5.7 before autoclaving. Culture conditions were 25°C and a photoperiod of 16 h with cool white light (Philips TL 85 fluorescent lamps, 36 μmol/m^{2}s). Plants developed from the axil of the nodal cuttings. The *in vitro* grown plants were subcultured every 4 weeks using nodal cuttings.

Explants and regeneration medium

Leaf explants were taken from 6 to 8 weeks old *in vitro* plants. Leaves were carefully removed from the stem using forceps, so that the axillary bud remained attached to the stem. Unless otherwise stated, the three youngest fully expanded leaf pairs (just below the apical meristem) were used. Leaves were cut across into two pieces and the half containing the base was incubated on regeneration medium. Occasionally, the axillary bud was taken along with the leaf explant. Shoots developing from axillary buds were scored after 1 week as 'escapes', and explants with axillary buds were excluded from the data.

To obtain petal explants, flower buds were removed two days before anthesis, sterilized as described above for nodal cuttings and petals were isolated aseptically. They were cut across

into two pieces and the half containing the base was incubated on regeneration medium.
For both the leaf and the petal explants a standard regeneration medium was used which consisted of MS-30 (pH 5.7) with 0.3 mg/l 6-benzyladenine (BA), 0.3 mg/l NAA and 0.7 % (w/v) agar (Oxoid).

Scanning Electron Microscopy
After 1, 3, 5, 8, 11 and 12 days on regeneration medium, leaf explants were collected for microscopic examination. They were fixed in 2 % (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) for 2 h at room temperature, rinsed three times for 30 min in distilled water, dehydratated through a graded ethanol series and critical point dried. The dried samples were examined with a Jeol ISM-35C Scanning Electron Microscope.

Plant age and leaf position
All leaves were taken from eight CPRO clones, donor plants of different ages (3, 5, 8, 10 and 12 weeks after subculture of nodal cuttings), and explants were examined for regeneration. The youngest leaf was numbered as position one. For each age 12 donor plants were studied.

Regeneration from leaves and petals
The regeneration capacity of 25 different cultivars was determined twice by incubating all leaves of 6 weeks old *in vitro* grown plants on the standard regeneration medium. The regeneration capacity of 55 different cultivars was tested twice using petals.

Transplantation into soil
In total, seven leaf regenerants from CPRO clone 89086 and a total of 20 leaf regenerants from eight other cultivars were analyzed in the greenhouse. Therefore, shoots were removed from the explants after 4 weeks culture on regeneration medium, and transferred to MS medium containing 0.1 mg/l NAA for root induction. After 8 weeks, plantlets were potted in a soil-peat mixture, covered with film for acclimatization, and subsequently transferred to the greenhouse.

Growth regulator concentrations
Using leaves of CPRO clone 89086, various combinations of BA and NAA concentrations were studied; 0, 0.1, 0.3, 0.9, 2.7 mg/l. The extreme concentrations of 0 and 2.7 mg/l BA or NAA were studied only once in two Petri dishes per treatment. Treatments of nine encompassing combinations of 0.1, 0.3, 0.9 mg/l BA and NAA were performed in two experiments with a total of at least seven replicate dishes per treatment. Regenerating percentages in the growth regulator experiment were analyzed according to a generalized linear model for binomial data. The average numbers of adventitious shoots per regenerating explants were analyzed according to a generalized linear model for Poisson counts (McCullagh and Nelder, 1989). Calculations were carried out by means of the computer program Genstat (Genstat 5 Committee, 1988).

Differences within a pair of leaves
While removing a pair of leaves from the stem, presence or absence of an axillary bud on the stem was recorded, as well as the sequence of removal (first or second). The influence of these factors on bud regeneration of the leaf bases was studied. The tested cultivars were clones 89086, 89117, 89091 and 89114, for which 129, 54, 36 and 36 pair of leaves were incubated respectively. Tests of independence were based on Pearson's X^2-statistic.

Optimum regeneration condition
To test the overall regeneration procedure, secondly removed leaf explants of clone 89086 were incubated on the optimum medium (MS-30 with 0.9 mg/l BA and 0.3 mg/l NAA), using ten replicate petri dishes, each with six leaf explants.

Assessment of results and statistical analysis
Unless stated, the regeneration experiment was carried out in a randomized block design and for every treatment four Petri dishes were used each containing six leaf explants derived from

the youngest three leaf pairs. Explants were observed weekly for newly formed primordia. After 4 weeks, regeneration capacity was determined as percentage of explants with adventitious shoots and as the mean number of adventitious shoots per regenerating explant. In the cultivar effect experiments, regeneration percentages and number of shoots formed were scored after 5 weeks, and in the petal regeneration experiments, after 8 weeks.

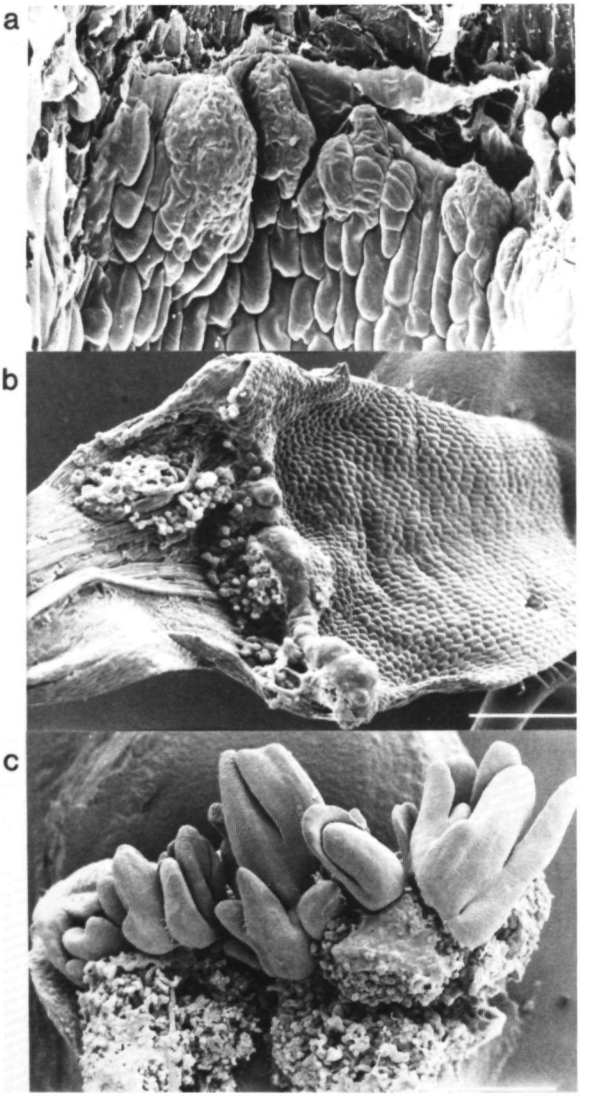

Figure 1. Scanning Electron Microscopy of development of adventitious shoots at the base of the leaves of CPRO clone 89086. (A) Day 5: cell divisions near wound surface. Bar = 100 μm. (B) Day 8: primordia were clearly visible. Bar = 1000 μm. (C) Day 11: developing shoots. Bar = 1000 μm.

Results

Adventitious shoot formation from leaf bases

Regeneration of shoots was observed on the base of leaf explants when incubated on MS medium with 0.3 mg/l BA and 0.3 mg/l NAA. Since regeneration was only observed if the base of the leaf was present, the best way to isolate the leaves was to remove them carefully from the stem with forceps, so that the axillary bud remained attached on the stem. Fig. 1 shows the development of adventitious shoots at the base of the leaves. After 5 days, cells near the wound surface started to divide; after 8 days, primordia were clearly visible and after 11 days, developing shoots were present. The new meristems were formed on the entire base of the explant. It is important to stress that the regeneration in this system is direct. The newly formed meristems develop directly from the parenchymatous cells and not from callus (data not shown). In fact only a limited amount of callus is formed under the conditions used. The regeneration procedure was optimized for a Diantini type (CPRO clone 89086) using *in vitro* grown plants.

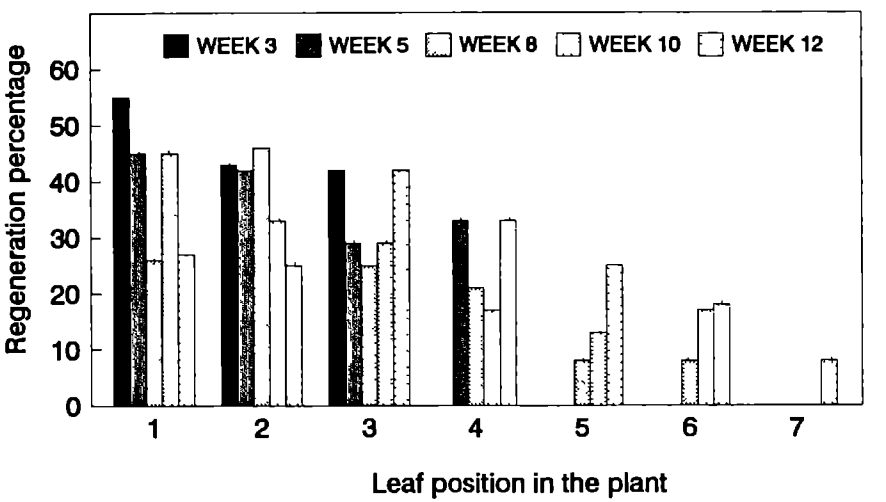

Figure 2. Effect of donor plant age and leaf position on regeneration percentage of *in vitro* leaf explants from carnation, CPRO clone 89086. The youngest leaf was numbered as position one. Error bars represent the standard error of the mean for 24 samples per treatment

Plant age and leaf position

Effect of the age of plant material as well as leaf position was tested in three different experiments for CPRO clone 89086. Figure 2 shows the results for regeneration percentage. The youngest leaves gave the best regeneration results, averaging 40% of regenerating explants with seven shoots per regenerating explant. Regeneration percentage decreased with increasing leaf age, although the age of the plant itself had no effect. In seven other tested cultivars, the effects of plant age and position of leaves corresponded to that observed in clone 89086 (data not shown). In addition to giving the best regeneration results, the youngest leaves had also the highest chance that the axillary bud remained attached at the leaf base.

Calculating all results of eight cultivars together, the mean frequency of 'escapes' decreased from 11% on top explants to 3% on bottom explants. The percentage of escapes was also dependent on plant age: using eight cultivars, mean percentage escapes of eight cultivars from explants of 3 to 12 weeks old plantlets varied respectively from 24 to 5%. The number of shoots growing from existing axillary buds generally increased when younger plant material was used, being effected both by the age of plant and the position of the leaves on the plant. In further regeneration experiments, only the three youngest leaves derived from 6 to 7 weeks old *in vitro* grown plants were used as starting material.

Regeneration from leaves and petals of 25 cultivars

Figure 3 shows the regeneration percentages of 25 cultivars, belonging to the major carnation types. The results were compared with the regeneration percentages obtained using petals as starting material. In all cases, adventitious shoot formation occurred without a callus phase, and at the base of the explants (both petals and leaves). Large differences in regeneration capacity have been observed between cultivars, and, within a cultivar, no correlation could be found between the regeneration capacity of leaves and petals.

Regeneration percentages varied between 0 and 42% for leaves, while for petals the maximal obtained regeneration percentage was 78%. However, in seven cases, regeneration took place for leaves but not for petals. Using leaf explants, all cultivars, except one miniature carnation (CPRO clone 89095), were able to regenerate; starting from petals, only 39 of the 55 tested cultivars were able to regenerate.

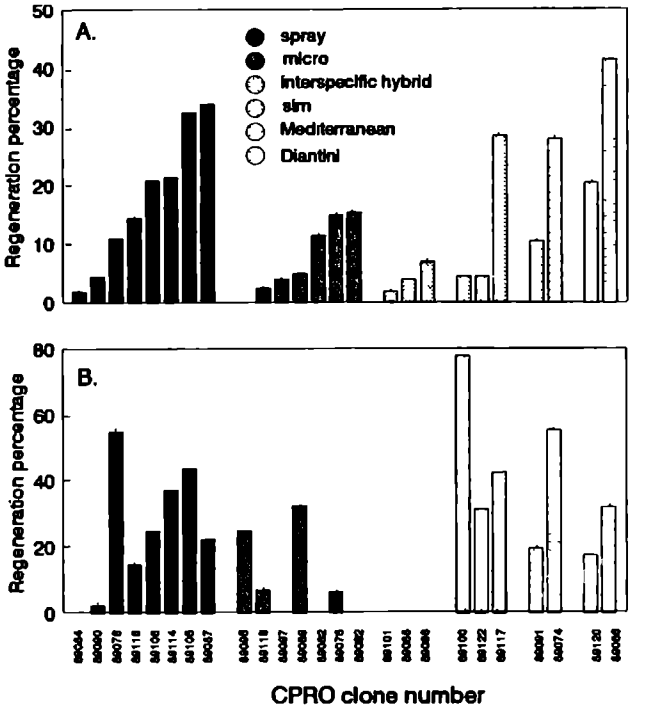

Figure 3. Regeneration percentages of *in vitro* leaf explants (A) compared with *in vivo* petals (B) in 25 cultivars of carnation. Per cultivar 48 leaf explants and 48 petal explants were used. Note that the y-axis in Fig. A is different from Fig. B.

Table 1. Effects of 6-benzyladenine (BA) and α-napthaleneacetic acid (NAA) at different concentrations (0.1, 0.3, 0.9 and 2.7 mg/l) on regeneration percentage of *in vitro* leaf explants from carnation (CPRO clone 89086).

NAA (mg/l)	BA (mg/l)				
	0	0.1	0.3	0.9	2.7
0	0	6 (6)	17 (6.8)	0	6 (1)
0.1	0	31 (3)	24 (4.8)	14 (11.6)	11 (23.5)
0.3	0	21 (8.6)	24 (6.7)	14 (10.9)	6 (2)
0.9	0	21 (3.9)	28 (6.1)	26 (7.2)	6 (2)
2.7	0	11 (4.5)	10 (4)	17 (4.5)	22 (11)

Mean number of adventitious shoots per regenerating explant is given between brackets. Results from the nine combinations of BA and NAA at 0.1, 0.3 and 0.9 mg/l are from two experiments with a total of at least 84 explants per treatment; results of the other combination are from 12 explants per treatment. Between combinations of NAA and BA at 0.1, 0.3 and 0.9 mg/l, percentages of regeneration were not different at $p = 0.05$.

Transplantation into soil

Shoots formed on leaf explants were easily transferred to rooting medium and subsequently to the greenhouse. The regenerants from leaves showed normal growth and plant morphology. However, the regenerated shoots from petals produced flowers prematurely *in vitro*, although the time of flowering varied between cultivars. For shoots from petals, it was often impossible to transfer them into soil, and only twice (CPRO clones 89086 and 89105) did regenerants show normal growth and flowering.

Hormone concentrations

To investigate if the regeneration percentages could be further increased, BA and NAA combinations were varied, using concentrations of 0, 0.1, 0.3, 0.9, 2.7 mg/l of both. No regeneration occurred on media containing only NAA. The highest numbers of explants showing adventitious shoot formation were observed in hormone combinations of 0.1, 0.3 and 0.9 mg/l of BA and NAA (Table 1), but differences between these treatments were not significant. In contrast, for the number of adventitious shoots, statistically significant effects of BA and NAA concentrations were found ($p<0.05$). The BA - NAA interaction is not significant and the effect of BA may be determined independently of that of NAA as seen in Table 2. The average number of adventitious shoots increased with increasing concentration of BA up to 0.9 mg/l, whereas for NAA the highest number was found at 0.3 mg/l.

Table 2. Effects of benzyladenine (BA) and α-naphthaleneacetic acid (NAA) at different concentrations (0.1, 0.3, 0.9 and 2.7 mg/l) on numbers of adventitious shoots regenerated on *in vitro* leaf explants from carnation (CPRO clone 89086).

Concentration of growth regulator	Mean number of adventitious shoots for	
	BA	NAA
0.1 mg/l	5.4 ± 0.80 *(a)*	5.8 ± 0.86 *(a)*
0.3 mg/l	6.3 ± 0.61 *(a)*	8.3 ± 0.86 *(b)*
0.9 mg/l	9.0 ± 1.17 *(b)*	5.8 ± 0.64 *(a)*

The effect of BA was determined independently of that of NAA, and the effect of NAA was determined independently of that of BA. Interaction between BA and NAA was not significant. Per treatment at least 256 explants were used.
Means within the same column followed by the same letter are not significantly different at the 5% level of probability according to a generalized linear model for Poisson counts (McCullagh and Nelder, 1989).

Response of a leaf pair

The regeneration response of the two leaves belonging to one pair was different, usually, only one showing regeneration. This effect was highly significant (Table 3) and to explain this phenomenon, a study was carried out to see whether a relationship existed between regeneration response and the presence of a bud in the leaf axils. In carnation the axillary buds of each leaf of a pair are not equally developed. In fact, in CPRO clone 89086, an axillary bud is only present in the axil of one of the leaves. Although the bud remains attached to the stem during the removal of the leaf (see Materials and methods), its presence might be indicative of a different physiological condition of the leaf explant influencing the regeneration capacity. Because such a relationship was not found, the sequence of removal of the two leaves of a pair was examined. Microscopical examination of the wounded surface of the leaves showed that much more of the tissue surrounding the stem was retained on the leaves removed secondly. There was a high correlation between sequence of leaf removal and regeneration response (Table 4). The second leaves yielded a regeneration response of 66%, compared to 5% for the first ones. The effect of removal sequence of leaves was also investigated in three other cultivars, a mediterranean type (CPRO clone 89091), a Sim type (CPRO clone 89117) and a spray carnation (CPRO clone 89114) and in all cases the explants from the leaf removed secondly gave significantly higher regeneration percentages (data not shown).

Table 3. Difference in regeneration capacity between the leaves of one pair in CPRO clone 89086.

	Percentage of the pairs with leaves regenerating			X^2
Position	0	1	2	
1	23	63	14	11.6
2	24	69	8	20.6
3	39	57	4	9.0

Position one, two and three was tested, with 112, 118 and 113 pair of leaves, respectively. Per pair the number of leaves showing regeneration was scored. Results are derived from five independent experiments. Differences were tested with X^2.

Table 4. Effect of (1) presence of a bud in the leaf axil and (2) sequence of leaf removal on regeneration capacity of carnation (CPRO clone 89086).

	Presence of an axillary bud		Sequence of leaf removal	
Results	Present	Absent	Firstly	Secondly
Obtained	38	52	6	84
Expected	45	45	45	45
X^2	1.09	1.09	33.8	33.8

In total 129 pair of leaves were tested; 90 leaves showed regeneration. Only three times did both leaves from one leaf pair show regeneration.

Optimum regeneration condition

Finally, the overall regeneration procedure was tested combining the optimum conditions. Secondly removed leaf explants incubated on the optimum medium, (MS-30 with 0.9 mg/l BA and 0.3 mg/l NAA), showed good regeneration (65% with 10.1 shoots per explant).

Discussion

In this article we have described a procedure for adventitious shoot formation on explants from leaves of *in vitro* grown plantlets. The procedure has been developed and optimized for CPRO clone 89086, a tetraploid of the Diantini type. The procedure leads to high regeneration percentages (up to 65%), and to high numbers of adventitious shoots per explant (over 10). It has been applied successfully to a wide range of cultivars belonging to all major carnation types. No correlation could be found between the regeneration capacity of leaves and petals of the various cultivars. Regeneration was more reliable using leaves and regenerated shoots could be counted after 4 weeks. Whereas in the case of petals, 8 weeks was needed and regenerants often showed 'early flowering', also seen with ovule regenerants (Kakehi,1979; Gimelli *et al.*, 1984). As shown earlier with *Begonia* (Takayama and Misawa, 1982), regeneration potential decreased with leaf age and only the first three leaf pairs down from the apex should be used.

The leaf removed secondly retained more material from the leaf base - stem transitional area and showed the highest regeneration percentage, the new

meristems being formed on the entire base of the explant (shown by Scanning Electron Microscopy).

A concentration of 0.9 mg/l BA and 0.3 mg/l NAA in the medium gave the highest average number of shoots per regenerating explant, although the regeneration percentage was not effected within the range of BA and NAA used. Somaclonal variation is expected to be low or absent since no intermediate callus phase took place and to-date all regenerants tested have shown normal growth and flowering.

The present regeneration method will be very useful for the development of gene transfer system based on *Agrobacterium tumefaciens* transformation.

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CHAPTER 3

IMPROVEMENT OF ADVENTITIOUS SHOOT FORMATION FROM CARNATION LEAF EXPLANTS

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Summary

Adventitious shoot formation from leaf explants of carnation (*Dianthus caryophyllus* L.) was investigated. The two leaves from one node of *in vitro*-grown plants showed different shoot-forming potential, depending on the order in which the leaves were removed from the stem. The leaf removed second formed more shoots and also had a large amount of adhering stem tissue. Explants with equal amounts of adhering stem tissue were obtained by making two incisions through the fused leaf bases, prior to their removal, resulting in an improved shoot formation. The procedure developed for leaf explants from *in vitro*-grown plants was also applied to leaf explants from greenhouse-grown plants. Shoot formation from leaf explants taken from greenhouse-grown plants was further improved by cutting the leaf explant longitudinally into two parts.

Introduction

Adventitious shoot formation for carnation (*Dianthus caryophyllus* L.) has been obtained from petals (Kakehi, 1979), axillary buds (Miller *et al.*, 1991), stems (Nugent, *et al.* 1991) and leaves from *in vitro*-grown plants (Van Altvorst, *et al.* 1992). The procedure described by Van Altvorst *et al.* (1992) allowed production of more than 10 adventitious shoots per explant with up to 65 % of the explants showing adventitious shoot formation. This procedure was also successfully applied to a wide range of cultivars belonging to all major carnation types. Since shoot formation was restricted to the base of the leaf, the best way to isolate the leaves was to carefully tear them from the stem using forceps while keeping the leaf base intact. The leaf that was removed second gave a much higher shoot regeneration response, possibly because of the larger amount of basal leaf tissue that remained attached to the leaf explant. In this paper, we further analyze the relation between shoot regeneration response and the adhering stem tissue. The shoot regeneration responses of leaf explants from *in vitro*- and greenhouse-grown plants were compared. The efficiency of adventitious shoot formation was further improved by modifying the preparation of the explants. The regeneration percentages of *in vitro* grown plants described in this article were low (33 %) compared to the results described in van Altvorst *et al.* (1992), in which the leaves removed second showed a regeneration

percentage of 65%. Although the regeneration percentages described here were low due to the different physiological conditions of the plants, the effects of the amount of stem tissue on regeneration efficiency were the same.

Material and methods

Explant isolation

The carnation plants (Diantini type, CPRO clone 89086) were obtained as rooted cuttings from a commercial source and were grown in a greenhouse at 16/14°C (day/night). Nodal cuttings from greenhouse-grown plants were surface-disinfected and cultured *in vitro* as described by Van Altvorst *et al.* (1992). Explants from greenhouse-grown cuttings and from *in vitro*-grown plants were taken from the three leaf pairs just below the apex. The two leaves of one pair were carefully removed from the stem using forceps in such a way that the axillary bud remained attached to the stem (Fig. 1A). The order in which the leaves of each pair (first and second) were removed from the stem was recorded and the explants were cultured on shoot regeneration medium.

Shoot regeneration medium

The shoot regeneration medium contained MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose (MS-30), 0.9 mg/l 6-benzyladenine (BA), 0.3 mg/l α-naphthaleneacetic acid (NAA) and 7 g/l purified agar (Oxoid) and was adjusted to pH 5.8 before autoclaving at 103 kPa (121 °C) for 20 min. Cultures were maintained at 25°C with a 16 h photoperiod (Philips TL 85 fluorescent lamps, 36 μmol/m^{2}s).

Assessment of results and statistical analysis

Experiments on the effect of explant preparation were carried out eight times with two petri dishes per experiment. All other experiments were carried out four times with two petri dishes each. Each petri dish contained the leaf explants from one carnation plant. Shoot regeneration percentages obtained in the experiments were analyzed according to a generalized linear model for binomial data (McCullagh and Nelder 1989). Calculations were carried out by means of the computer program Genstat (Genstat 5 Committee, 1988).

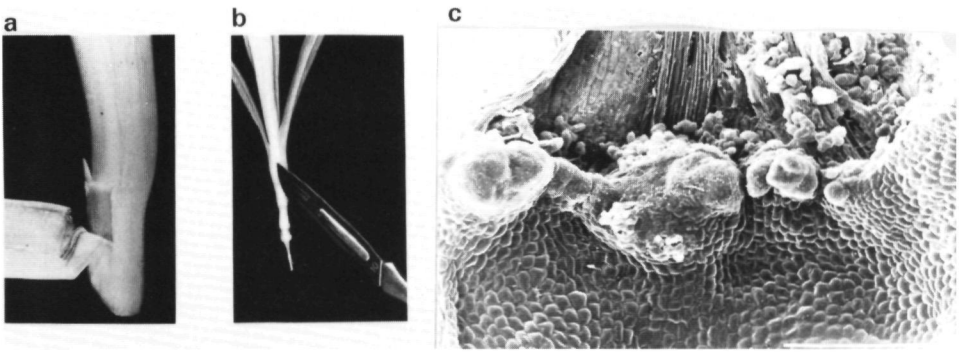

Figure 1. Isolation of leaf explants of greenhouse-grown carnations (CPRO clone 89086). (A) Leaf was carefully removed from the stem using forceps while the axillary bud remained attached to the stem. (B) A lengthwise incision was made through the fused leaf base, prior to removal of the leaves with forceps. (C) Scanning electron micrograph of developing adventitious shoots at the base of a leaf from an *in vitro*-grown plant (CPRO clone 89086) after 8 days. Bar = 1000 μm.

Results

Response of a leaf pair

Shoot formation was observed at the base of leaf explants when incubated on MS-30 medium supplemented with BA and NAA. After 8 days, active cell division had started in the basal region of the leaf explant, as shown in Fig. 1C. New meristems were formed along the entire base of the explant. For *in vitro*-grown plants, the shoot regeneration response of the two leaves belonging to one leaf pair was different, the leaves that were removed second showing a much higher shoot regeneration frequency (33%) than the leaves that were removed first (6%). In addition, the mean number of adventitious shoots per regenerating explant was higher, 8 and 5 respectively. Due to coalescence of the bases of each pair of leaves, the leaf removed first contained only a small amount of adhering stem tissue and a relatively small leaf base. Consequently, the leaf removed second contained more leaf tissue, resulting in a wider leaf base and a larger amount of adhering stem tissue, as illustrated in Fig. 2 (treatment a1 + a2). Such a difference in shoot formation between two leaves of one pair was not observed when greenhouse-grown plants were used. Compared to *in vitro*-grown plants, greenhouse-grown plants were larger and had thicker leaves. Removal of such leaves resulted in explants with only minor amounts of adhering stem tissue (Fig. 2, treatment A1 + A2). Small, but equal, amounts of adhering stem tissue were present on the leaves of each pair and shoot regeneration percentages were the same, 21% with a production of about 8 shoots per explant.

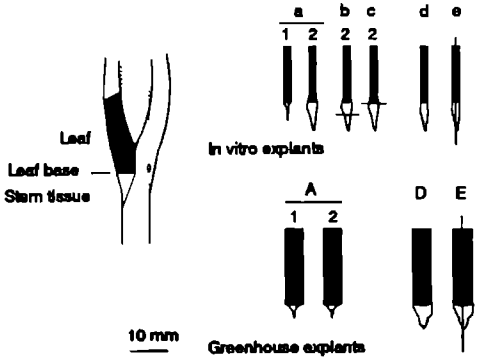

Figure 2. Scheme for the preparation of leaf explants from *in vitro*-grown plants (a,b,c,d,e) and from greenhouse-grown plants (A,D,E). Leaves were removed from the stem (1 = leaf removed first, 2 = leaf removed second) as described in the text.

Size of attached stem tissue

To test the effect of the size of the attached stem tissue, it was cut off either partly (Fig. 2, treatment b) or completely (Fig. 2, treatment c). No effect on shoot regeneration frequency or on the number of shoots per explant was observed after partly removing the tissue (data not shown). However, complete removal of the adhering stem tissue resulted in a reduction in shoot formation from 30% to 16%.

In order to control the amount of stem tissue, lengthwise incisions on either side of the stem were made prior to the removal of the leaves (Fig. 1B). In this way, the two leaf explants from one pair were made identical (Fig. 2, treatment d+D). This treatment improved the shoot formation for leaves both from *in vitro*- and greenhouse-grown plants (Table 1). Shoot regeneration percentages were enhanced from 21% to 57% for explants derived from *in vitro*-grown plants, and from 21% to 47% for greenhouse explants.

Table 1. Effect of different types of incisions on shoot formation from leaf explants.

	Explants from *in vitro*-grown plants			Explants from greenhouse-grown plants		
Treat-ment	Shoot regeneration (%)	Number of shoots per explant[1]	Number of shoots per cutting[2]	Shoot regeneration (%)	Number of shoots per explant[1]	Number of shoots per cutting[2]
A/a	21 ± 4	9 ± 2	12	21 ± 4	10 ± 2	12
d/D	57 ± 5	7 ± 1	24	47 ± 5	12 ± 1	32
e/E	18 ± 4	11 ± 2	23	60 ± 5	13 ± 1	90

For all treatments, the mean shoot regeneration percentage of both the first as well as the second removed leaf is given. Each value is a mean ± s.e. of 16 petri dishes.

1: Mean number of adventitious shoots per regenerating explant.

2: The total number of shoots per cutting was calculated by 'shoot regeneration percentage' x 'mean number of shoots per explant' x 'number of explants per cutting (6 explants for treatment A/a and D/d; 12 for treatment E/e)'.

Optimum explant isolation

Finally, the effect of another type of incision was analyzed. After removal of the leaves, an incision was made longitudinally through the center of the leaves (through the main vein), resulting in two equal parts (Fig. 2, treatment e+E). For explants from *in vitro*-grown plants, this extra incision decreased the shoot

regeneration percentage, but explants from greenhouse plants showed a higher shoot regeneration frequency (Table 1). Moreover, the total number of adventitious shoots per cutting was further increased. Using this improved procedure, approximately 90 adventitious shoots were obtained from the first three leaf pairs from each cutting, whereas in the controls, where the original procedure was used, only 12 shoots per cutting were obtained.

Discussion

The results presented here show that the leaf base shoot regeneration procedure can be applied to both *in vitro*-grown and greenhouse-grown plants. In both cases, adventitious shoots developed at the base of the leaf explants. Apparently, the base of the explants contains the shoot regeneration competent cells, a phenomenon also observed for carnation petals (Van Altvorst *et al.*, 1992), cotyledons of white spruce (Toivonen and Kartha, 1988) and cucumber hypocotyls (Gambley and Dodd, 1991). The leaves removed second from *in vitro*-grown plants always showed a much higher shoot regeneration frequency. As a result of the isolation procedure, a larger amount of stem tissue remained attached to these leaves. Removal of adhering stem tissue resulted in a decrease in shoot formation, showing that its presence stimulated adventitious shoot formation at the transition zone of stem and leaf base. The stem tissue could be necessary for nutrient uptake, or it could provide other factors beneficial for shoot formation. Gambley and Dodd (1991) showed that some intact cotyledon was required for shoot production at the base of cucumber cotyledons and suggested that a substance produced in the cotyledon was transported to its base where it induced shoot production.

The removal of the leaves from the stem could be improved by making longitudinal cuts through the stem at the fused region of the leaves. For *in vitro*-grown plants this treatment resulted in explants with equal amounts of adhering stem tissue. Culturing such explants on shoot regeneration medium resulted in a three-fold enhancement of the shoot regeneration frequency. When such incisions were made in greenhouse-grown plants, the leaf explants also had a larger amount of adhering stem tissue and shoot formation was considerably improved. For explants from greenhouse-grown plants, the shoot formation could further be optimized by enlarging the wound surface of the explants. An eight-fold improvement was obtained by combining both pretreatments. Such a

treatment, using explants from *in vitro*-grown plants, however, resulted in a strong decrease in shoot formation. This was possibly because these explants were smaller and therefore more sensitive to the wounding.

The results presented here show that the shoot regeneration procedure previously described for leaf explants of carnation can be improved considerably by proper preparation of the explants. For greenhouse-grown plants, both the presence of adhering stem tissue and the amount of wound surface enhance the number of adventitious shoots. Although the mechanisms involved are not well understood, it is tempting to postulate that the supply of nutrients, both from the adhering stem tissue and from the medium via the wounded surface, is a key factor in the improvement of shoot formation in carnation leaf explants.

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CHAPTER 4

CELLS WITHIN THE NODAL REGION OF CARNATION SHOOTS EXHIBIT A HIGH POTENTIAL FOR ADVENTITIOUS SHOOT FORMATION

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(submitted)

Summary

Adventitious shoot formation was studied with leaf, stem and axillary bud explants of carnation (*Dianthus caryophyllus* L.). The shoot regeneration procedures were applicable for a wide range of cultivars and shoot regeneration percentages were high for all explant types. Using axillary bud explants, shoot regeneration efficiency was independent on the size of the bud and on its original position in the plant. In contrast, shoot regeneration of stem and leaf explants was strongly dependent on their original position in the plant. The youngest explants, taken from stem and leaves, showed the highest level of shoot regeneration. The adventitious shoot primordia developed at the periphery of the stem segment and at the base of leaf explants. Both in axillary bud, stem and leaf explants, shoot regeneration originated from node cells, located at the transition area between leaf and stem tissue. Moreover, a gradient in shoot regeneration response was observed, increasing towards the apical meristem.

Introduction

Adventitious shoot formation in carnation has been described for petal explants (Kakehi, 1979; Gimelli *et al.*, 1984), but vitrification and premature flowering of the shoots was a severe problem (Leshem, 1986; Van Altvorst *et al.*, 1992). Adventitious shoot formation has also been reported for axillary bud explants (Miller *et al.*, 1991) and stem explants (Nugent *et al.*, 1991). As part of our research program to develop an *Agrobacterium*-mediated gene transfer system in carnation, we have developed a shoot regeneration procedure using leaf explants (Van Altvorst *et al.*, 1992). It was shown that shoot regeneration was restricted to the base of the leaf. The efficiency of adventitious shoot formation from leaf explants of carnation has been further improved by some modifications in the explant isolation method (Van Altvorst *et al.*, 1994). Similarly to our results, shoot regeneration of leaf explants was also described recently by Messeguer *et al.* (1993).

A prerequisite for the production of transformed plants is the availability of an efficient method to regenerate a plant from a transformed cell. To analyze which shoot regeneration system will be the most suitable for *A.tumefaciens*-mediated transformation, we have compared the shoot regeneration procedures for leaf,

stem and axillary bud explants. Because *A.tumefaciens* susceptibility is often dependent on the cultivar used (for a review, see Van Wordragen and Dons, 1992), we tested the three mentioned shoot regeneration procedures for a range of cultivars, belonging to various carnation types. The shoot regeneration procedures have been further analyzed in order to enhance the shoot regeneration frequency. The results presented suggest that the same cell layers were responsible for initiation of shoot formation in three explant types.

Material and methods

Plant material

Twenty five cultivars of carnation (*Dianthus caryophyllus* L.) were used, which are indicated with CPRO-clone numbers and belong to various carnation types: Mediterranean, Sim, Spray, Diantini, micro carnations and interspecific hybrids. Plants were obtained as rooted cuttings from commercial sources and grown in the greenhouse at 16/14°C (day/night). Three weeks old greenhouse grown cuttings were used as starting material for the isolation of leaf and stem explants, and five weeks old cuttings were used for axillary bud explants. Cuttings were surface sterilized and maintained *in vitro* as described by Van Altvorst *et al.* (1992). For the isolation of *in vitro* leaf explants, six weeks old *in vitro* plants were used.

Explant isolation

Nodes are slightly enlarged portions of the stem where leaves and buds arise and side branches originate. Fig. 1a shows the different node positions in a three weeks old carnation cutting and the various organs are depicted schematically in Fig. 2. The youngest node was visible just below the apical meristem (upper arrow, Fig. 1a). The leaves from the first node were the youngest leaves used in shoot regeneration experiments (and marked as position 1), although the next leaves were already initiated from the apex. Leaf explants were isolated from the youngest 3 nodes, as described by Van Altvorst *et al.* (1992, 1994). The leaves were carefully torn from the stem, as indicated by the dotted line in Fig. 1b.

After removal of leaves and axillary buds, the youngest two internodes were isolated using a binocular. Because the upper internode (marked as position 1) was not yet elongated, explants from this internode were 1-2 mm in size, whereas explants from the second internode were 3-4 mm in size. Both internode one and two were divided into two parts, an upper explant (explant 'α') and a lower explant (explant 'β').

Using a binocular, axillary buds with a size of 0.5 to 5 mm were isolated from the youngest five nodes. Axillary buds are composed of relatively small, non-vacuolated cells (Fig. 1c). In most carnation cultivars used, only one of the two leaf axils contained an axillary bud. Longitudinal section of carnation nodes showed the presence of small cells in the leaf axil while axillary buds were absent (Fig. 1b).

Histological examination

The localization of nodes was analyzed in three weeks old carnation cuttings. The cuttings were longitudinally hand-sectioned with a razor blade under a stereomicroscope into 0.5- to 1.0 mm sections (Fig. 1a). Dark-field photographs (Fig. 1b and 1c) were taken from detailed sections using a Zeiss microscope.

Shoot regeneration media and incubation conditions

Murashige and Skoog (1962) basal medium (MS-30) with trypton (0.5 g/l), sucrose (30 g/l) and Oxoid purified agar (8 g/l) was used, supplemented with various growth regulators. The pH was adjusted to 5.8 prior to autoclaving at 121 °C for 20 minutes. Leaf explants were incubated on

medium containing 0.9 mg/l 6-benzyladenine (BA) and 0.3 mg/l α-naphthaleneacetic acid (NAA), axillary bud explants on medium containing 3 mg/l BA and 1 mg/l NAA (Miller *et al.*, 1991) and stem explants on medium containing 2 mg/l BA and 1 mg/l NAA (Nugent *et al.*, 1991). All cultures were grown at 25°C with a photoperiod of 16 h light / 8 h darkness (Philips TL 85 fluorescent lamps, 36 μmol/m^{2}s).

Assessment of results

Occasionally, the axillary bud was taken along with leaf or stem explants. After one week, explants with developing axillary buds were marked. These marked explants were excluded from the analysis. After four weeks, the shoot regeneration capacity was determined as percentage of explants with adventitious shoots (shoot regeneration percentage) and the mean number of adventitious shoots per regenerating explant. Calculations were carried out by means of the computer program Genstat (Genstat 5 Committee, 1988).

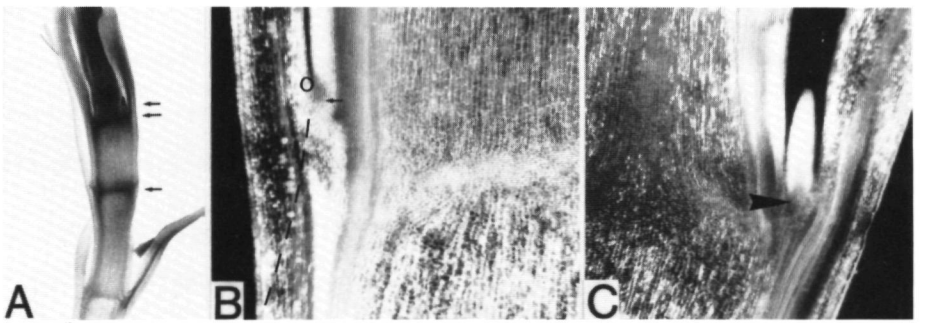

Figure 1. Light microscopy of longitudinal sections through a 3 weeks old greenhouse cutting. (A) A node with the successive node positions. Arrows show the sequence of the node positions. (B) A leaf axil without axillary bud, showing the presence of small cells (arrow) on the transition area from leaf to stem. The dotted line shows the wound surface of the leaf explant that results from removing from the stem. The black circle indicates the origin of shoot regeneration in leaf explants. (C) A leaf axil with axillary bud (arrow).

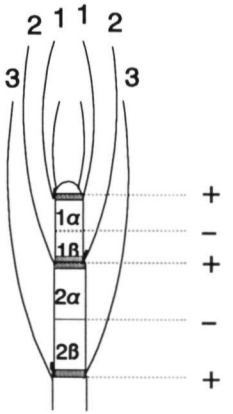

Figure 2. A schematic presentation of a longitudinal section through a carnation shoot illustrating the subsequent leaf and stem positions. Although successive pairs of leaves occur at right angles to one another, all pairs have been represented in the same plane for this illustration. The dark shaded area (indicated with '+') depicts the wound surface after cutting through the node. The other wound surfaces are indicated with '-'.

Results

Shoot regeneration from leaf explants

Fig. 1a shows the different node positions in a three weeks old carnation cutting and the various organs are depicted schematically in Fig. 2. Development of adventitious shoots on leaf explants took place without an intermediate callus phase (Van Altvorst *et al.*, 1992). These shoots developed at the base of leaf explants (Fig. 3a) and started from subepidermal cells (results not shown), indicated by a black circle in Fig. 1b. The formation of adventitious shoots occurred along the entire tranverse wound surface and was not restricted to cells in the leaf axil.

The effect of the original position of the leaves in carnation plants on the shoot regeneration capacity was determined by isolating leaf explants from the youngest three nodes from *in vitro* plants. Leaves derived from the youngest node (position 1) showed the highest shoot regeneration response (Fig. 4a). Leaf explants were also isolated from greenhouse cuttings, and again, the youngest leaf explants showed the highest shoot regeneration capacity (Fig. 4b).

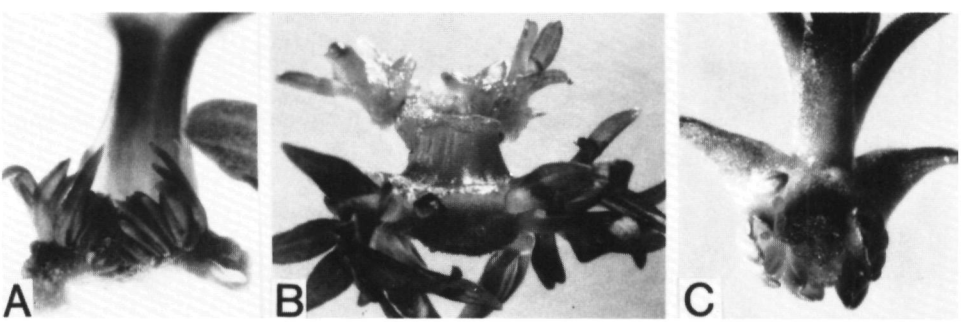

Figure 3. Shoot regeneration of different explant types. (A) Adventitious shoot formation at the base of a leaf explant, 18 days after incubation. (B) Shoot regeneration from a stem explant, 18 days after incubation. In this explant, shoot regeneration occurred at the periphery of both wound sites. (C) Shoot regeneration from an axillary bud explant, 14 days after incubation. At the base of the bud a circle of several adventitious shoots emerged.

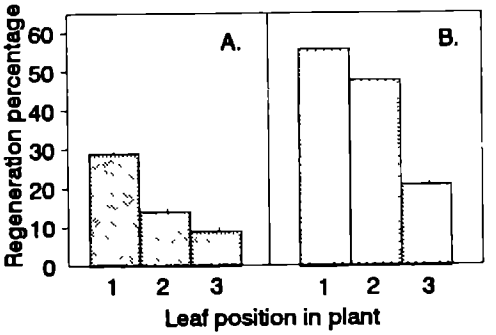

Figure 4. Effect of explant position on the shoot regeneration percentage of leaf explants. (A) Leaf explants derived from *in vitro* plants. Results are based on 4 explants per position in 25 cultivars (B) Leaf explants derived from greenhouse cuttings. Results are based on at least 6 explants per position in 10 cultivars.

Shoot regeneration from stem explants

Stem explants were incubated on shoot regeneration medium and within two weeks, adventitious shoot primordia became visible at the periphery of the stem segment (Fig. 3b). The development of adventitious shoots often started in an annular zone (results not shown). The shoot regeneration capacity of stem explants was determined for 17 cultivars (Fig. 5a). Fourteen cultivars produced adventitious shoots, with shoot regeneration frequencies between 4 and 79 %, and the mean number of adventitious shoots per regenerating explants varied from 1 to 18 (results not shown). A correlation of 0.95 was found between the shoot regeneration percentage and the mean number of adventitious shoots. The large variation in shoot regeneration capacity observed between cultivars could not be related to the carnation type (Fig. 5a). As observed for leaves, the shoot regeneration from stem explants was dependent on their original position in the plant (Table 1). The upper explant from the first internode showed the highest shoot regeneration percentages, averaging 56 % of regenerating explants with 11 shoots per regenerating explant, and gradually decreased in the next explants.

To analyze if the origin of shoot regeneration was restricted to the nodal region, stem explants were isolated from 26 cuttings (CPRO 89086) and the number of the adventitious shoots was determined for the different wound surfaces. The results are presented in Table 2 and show that shoot regeneration, both in percentage as in the number of shoots per explant, is higher in wound surfaces obtained by cutting through the node. This effect was very evident in explants of the older internode (position 2).

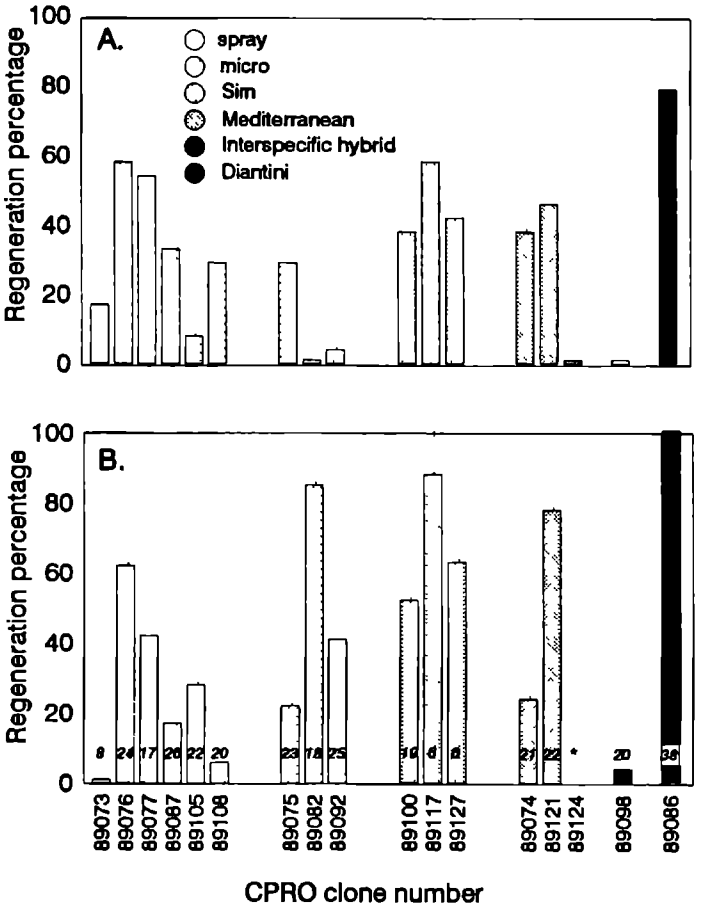

Figure 5. Shoot regeneration percentages of stem explants (A) and axillary bud explants (B) in 17 cultivars. Per cultivar 48 stem explants were incubated and all axillary bud explant with a size of 1 to 5 mm in 10 cuttings. The number of buds which could be isolated from 10 cuttings is given in the vertical bar. Only one explant was obtained from ten CPRO 89124 cuttings, it was not possible to show an accurate shoot regeneration response (asterisks).

Shoot regeneration from axillary bud explants

Because of the small size (mostly 0.5 - 1 mm), the isolation of axillary bud explants was difficult and time-consuming in contrast to e.g. the isolation of leaf explants. The number of explants which could be isolated from 10 cuttings was strongly dependent on the cultivar (Fig. 5b). For 17 cultivars, the shoot regeneration capacity was tested by incubating axillary buds from the youngest five nodes varying in size of 0.5 to 5 mm. During culture, the axillary bud developed into a shoot, but at the cut surface of the bud a circle of several adventitious shoots emerged (Fig. 3c). The shoot regeneration frequency varied

considerably between cultivars and was influenced by the variation in the number of axillary buds per cultivar that could be isolated (Fig. 5b). The maximum shoot formation was observed for CPRO clone 89086 (shoot regeneration capacity of 100 % in 38 explants). For CPRO 89124, only one explant was isolated from 10 cuttings. The shoot regeneration capacity of this clone could therefore not be determined.

Three Sim type cultivars (CPRO clone 89100, 89117 and 89132) were used to investigate the effect of size and position of axillary bud on shoot regeneration percentage. Axillary buds were isolated from the upper three nodes from 40 cuttings for each cultivar. Most of the buds were 0.5 to 1 mm long, although there was no evidence that the size of the bud influenced the development of shoots (data not shown). The shoot regeneration percentages were 83 to 91 % and were, in contrast to leaf and stem explants, not dependent on the original position of the axillary bud on the plant (data not shown).

Table 1. Effect of stem explant position on adventitious shoot formation. Two explants were derived from the youngest two internodes (α=upper stem explant, ß=lower stem explant). Per position results (± s.e.) are shown from 108 explants derived from 18 cultivars in three independent experiments.

Internode	Explant position	Shoot regeneration percentage	Mean number of adventitious shoots
1	α	56 ± 3	11.1 ± 0.8
1	ß	27 ± 3	9.7 ± 1.0
2	α	26 ± 3	6.0 ± 0.8
2	ß	14 ± 3	5.3 ± 1.1

Table 2. Effect of the wound surface obtained by cutting through the node in stem explants from CPRO 89086 on the shoot regeneration percentage and mean number of adventitious shoots. Values (± s.e.) are the mean of 52 wound surfaces. For each stem explant, the wound surface resulting from cutting through the node (+) and the other wound surface (-) were considered separately.

Internode	Wound surfaces	Shoot regeneration percentage	Mean number of adventitious shoots
1	+	73 ± 5	10.6 ± 0.9
1	-	60 ± 6	5.8 ± 0.8
2	+	69 ± 5	5.7 ± 0.6
2	-	14 ± 4	2.5 ± 0.8

Discussion

The adventitious shoot regeneration on stem, leaf and axillary bud explants, as described in this article, was located in the leaf axil and the youngest explants showed the highest level of shoot regeneration. The adventitious shoot primordia were initiated on the periphery of the stem segment, indicating that meristematic activity was located in the transition area between leaf and stem tissue. This agrees well with former observations by Dommergues and Gillot (1973) and Custers (1978). After decapitation of the carnation main shoot in greenhouse-grown plants, numerous shoots developed in annular zones extending between axils of opposite leaves. This bud formation was restricted to young internodes. Custers (1978) suggested that these buds were adventitious.

Shoot regeneration of axillary bud explants was independent on their position on the plant, whereas shoot regeneration of stem and leaf was dependent on the original plant position, and was higher in younger parts. Also within a leaf, such a gradient in response was observed, shoot regeneration was mainly restricted to the base of the explant. Concerning young stem explants, both nodal and internodal explants were able to produce adventitious shoots, whereas shoot regeneration from older stem explants was mainly restricted to the node. Histological observation showed the presence of specific cells at the transition zone from stem to leaf. These cells might be responsible for the initiation of adventitious shoots. Further histological studies are needed to prove the accurate location and origin of meristematic cells.

Leaf primordia are formed at regular intervals and the stem is divided by nodes at the points of leaf insertion. Histological studies of *Dianthus caryophyllus* revealed that the strongly developed intercalary meristem and the wave of maturation is reminiscent of grasses (Sushan and Johnson, 1955). In leaves of grasses, cell divisions cease first in the distal portions of the lamina, but continue at the leaf base much longer (for a review, Dale, 1988). Sushan and Johnson (1955) observed that the same holds for mature carnation leaves with basal regions completely meristematic. We have shown here that shoot regeneration of carnation leaf explants was restricted to the base and that a developmental gradient exists for adventitious shoot formation, the youngest explants (just below the apex) showed the highest level of shoot regeneration. All these results strongly suggest that cell differentiation in carnation tissues leads to a decrease of the shoot regeneration capacity.

In carnation, the nodes are produced in close succession at the shoot apex and become separated by the development of internodes. This phenomenon varies in intensity, timing and degree of localization of the actively dividing region. In the youngest internode, all cells actively divide, but later the meristematic activity becomes confined to a more or less restricted region, the base of the internode (Sushan and Johnson, 1955). This phenomenon might explain that internode cells of the first internode are also regenerating at internode surfaces, while internode cells of the second internode were less regenerable (Table 2).

Champagnat and Berthier (1957) described that in Caryophyllaceae (the family to which carnation belongs) not all axils contained an axillary bud. But, no relation existed between shoot regeneration of leaf explants and the presence of an axillary bud in the leaf axil (Van Altvorst *et al.*, 1992). Histological studies by Sushan and Johnson (1955) showed no discernible differences between young and older axillary buds of carnation. This agrees well with the observation that shoot regeneration of axillary buds was not dependent on the position of the axils in the plant. Also the size of the isolated meristems did not influence the mean number of adventitious shoots. This contrasts with results of Miller *et al.* (1991), who showed a linear decline in shoot regeneration with increasing bud size. The different results might be explained by the fact that in our research greenhouse-grown plants were used for the isolation of axillary bud explants, while Miller *et al.* (1991) have used tissue-cultured plants.

Efficient adventitious shoot formation was obtained both from leaf base, stem and axillary bud explants. All shoot regeneration procedures gave high regeneration percentages and could be applied to a wide range of cultivars belonging to all major carnation types (for leaf explants, see Van Altvorst *et al.*, 1992). Large differences in shoot regeneration capacity have been observed between cultivars. Within a cultivar, no correlation could be found between the shoot regeneration capacity of leaf, stem and axillary bud explants. For most cultivars, the shoot regeneration percentage was highest for axillary bud explants.

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CHAPTER 5

GENETIC TRANSFORMATION OF CARNATION (*DIANTHUS CARYOPHYLLUS* L.) BY *AGROBACTERIUM RHIZOGENES*

A.C. van Altvorst, T. Riksen-Bruinsma and J.J.M. Dons

Summary

Carnation (*Dianthus caryophyllus* L.) leaf explants were infected with *Agrobacterium rhizogenes* containing two plasmids, the wild type Ri-plasmid and the binary plasmid p35SGUSint. The T-DNA of the binary plasmid contained the neomycin phosphotransferase (*nptII*) gene and the ß-glucuronidase (*gus*) gene. Genetic transformation was demonstrated by expression of the *gus* gene, measured by the number of GUS-positive spots. In this way, various cultivars of carnation were tested for their susceptibility to the wild type *A.rhizogenes* strain LBA9402. Various factors were analyzed that influenced the DNA-transfer of *A.rhizogenes* to leaf explants. Leaf explants derived from greenhouse-grown plants were more susceptible for DNA transfer compared to leaf explants derived from *in vitro*-grown plants. Besides, in the plant there was a gradient for *A.rhizogenes* susceptibility, the youngest leaf explants showed the highest number of GUS-positive spots. Six months after callus initiation, calli still showed blue staining indicating that genes were stably incorporated and expressed, but it was not possible to develop transgenic plants from such cultures.

Introduction

The cutflower carnation (*Dianthus caryophyllus* L.) is an economically important ornamental crop for which molecular breeding strategies would be very useful. Of all known gene transfer methods, the *Agrobacterium*-mediated transformation is the most efficient way to introduce foreign genes into dicotyledonous plants. *Agrobacterium* is a plant pathogenic soil bacterium that infects wounded plant cells, leading to the induction of tumorous growth on the infected spot. There are two tumorigenic species of *Agrobacterium*; *A.tumefaciens*, that induces crown galls and *A.rhizogenes*, that induces the hairy root disease, an uncontrolled proliferation of highly branched roots with many root hairs (reviewed by Birot *et al.*, 1987). Defined regions of DNA (T-DNA), from Ti (tumour-inducing) and from Ri (root-inducing) plasmids are transferred into plant cells and integrated into plant DNA. The T-DNA carries phytohormone genes which are expressed in transformed plant cells and which lead to tumour and hairy root formation respectively (for a review, see

Zambryski *et al.*, 1989). Both *Agrobacterium* species can be used for transformation. The onco-genes present in the T-DNA of *A.tumefaciens* lead to an overproduction of hormones, which is incompatible with normal regeneration. Therefore disarmed plasmid are being used. In contrast, hairy roots resulting from the integration of the wild-type Ri T-DNA can regenerate in whole plants as observed for many species (reviewed by Tepfer, 1990). A disadvantage is that regeneration leads to aberrant plants; they frequently show alterations in leaf morphology, internode length, root geotropism, flower morphology and plant generation time. This problem can be overcome by using a 'binary system', a *A.rhizogenes* strain with both the Ri-plasmid and a vector plasmid carrying a T-DNA with selectable marker genes and the gene of interest. Both T-DNA's might integrate in the plant genome at different locations and might segregate in the next generation (Shahin *et al.*, 1986). *A.rhizogenes* is infectious on a high number of plant species (reviewed by Porter, 1991) and its interaction with carnation has been described previously by Mugnier *et al.* (1988). Hairy roots developed on carnation stem explants after inoculation with strain A4. In preliminary experiments with nine carnation cultivars we observed that gene transfer (as measured by the number of GUS-positive spots after infection) by *A.rhizogenes* was about 20 times more efficient than *A.tumefaciens* transformation. For the development of a genetic transformation protocol for carnation we have used the agropine *A.rhizogenes* strain LBA9402 into which a binary plasmid was introduced, p35SGUSint. The plasmid p35SGUSint contained the reporter gene *gus* (Jefferson *et al.*, 1987), and the *nptII* gene to provide kanamycin resistance as a selectable marker. The *gus* gene contained an intron derived from potato gene LS1 (Vancanneyt *et al.*, 1990), inhibiting bacterial GUS activity. This feature together with the high sensitivity of the histochemical staining procedure allows the detection of single transformation events soon after *Agrobacterium* infection without interference of bacteria which are still present. Transformations were performed on leaf explants which showed efficient regeneration on many carnation cultivars (Van Altvorst *et al.*, 1992). Based on the number of GUS-positive spots, various cultivars of carnation were tested for their susceptibility to the wild type *Agrobacterium rhizogenes* strain LBA9402 and various factors were analyzed that influenced DNA-transfer of *A.rhizogenes*. Stable transformed callus was obtained after infection of greenhouse leaves with LBA9402(p35SGUSint). In order to obtain transgenic plants from this callus, we tested a range of NAA and zeatin concentrations. A combination of these hormones was successfully

applied for regeneration of (non-transformed) callus in several *Dianthus* species (Nakano and Mii, 1992).

Material and methods

Plant material, explants and regeneration medium
Several carnation cultivars were obtained as rooted cuttings from commercial sources and grown in the greenhouse at 16/14 °C (day/night). The cultivars are indicated with CPRO clone numbers (as mentioned in Fig. 4). *In vitro*-grown plants were vegetatively propagated as described by Van Altvorst *et al.* (1992). Leaf explants were derived from six weeks old *in vitro*-grown plants or from three-four weeks old greenhouse cuttings. Explants were excised as described by Van Altvorst *et al.* (1992), infected with *Agrobacterium* and, unless otherwise mentioned, incubated on shoot regeneration medium (SRM): MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose (MS-30) with 0.5 g/l trypton, 0.9 mg/l 6-benzyladenine (BA), 0.3 mg/l α-napthaleneacetic acid (NAA). Sterile cuttings of tobacco (*Nicotiana tabacum* 'Petit Havana SR1') were grown on MS medium with 2 % sucrose and without hormones. Explants of four weeks old plantlets were used in transformation experiments.

Bacterial strains
A.rhizogenes strain LBA9402 is an agropine strain harbouring the wild type pRi1855. The binary vector p35SGUSint (Vancanneyt *et al.*, 1990) was introduced in LBA9402 by tri parental mating. LBA9402(p35SGUSint) was cultured in YMB medium (1.0 % mannitol, 0.05 % K_2HPO_4, 0.04 % yeast extract, 0.02 % $MgSO_4.7H_2O$, 0.01 % NaCl, pH 7.0), supplemented with 100 μM acetosyringone, 100 mg/l rifampicin and 50 mg/l kanamycin. Bacteria were cultured overnight at 28 °C. Before inoculation the O.D. at 550 nm was adjusted to 0.1 with liquid SRM medium with 100 μM acetosyringone.

Transformation
Explants were immersed in the bacterial suspension for 10 minutes and cocultivated for 2 days on SRM with 100 μM acetosyringone. After cocultivation, explants were blotted dry with sterile Whatman No. 1 filter paper and placed on selection medium that consisted of SRM supplemented with 200 mg/l carbenicillin and 300 mg/l vancomycin, supplemented with 30 or 100 mg/l kanamycin. For selection with leaf explants from *in vitro-grown* plants a concentration of 30 mg/l kanamycin was used, whereas for selection with greenhouse leaf explants 100 mg/l was used. Every four weeks, explants were transferred to fresh selection medium. The amount of callus and root growth was scored using an arbitrary scale from - (no calli/roots) to + (minor development of calli/roots) and + + (large number of calli/roots).
As a positive control, tobacco leaf explants were transformed according to the leaf-disk transformation method described by Horsch *et al.* (1985).

Histochemical GUS assay
ß-Glucuronidase (GUS) activity of transformed plant cells was determined by a histochemical assay. The GUS assay was performed essentially as described by Jefferson *et al.* (1987) using a modified extraction buffer containing: 100 mM sodium phosphate buffer (pH 7.5), 10 mM Na_2-EDTA (ethylene diamine tetra acetic acid), 0.025 % (v/v) thimerosal, 0.1 % (v/v) Triton X-100. To 100 ml of this buffer 1 ml ironcyanide solution (21.1 mg/ml potassium ferricyanide and 16.5 mg/ml potassium ferrocyanide in H_2O) and 2.5 ml X-Gluc solution (20 mg/ml 5-bromo 4-chloro 3-indolyl ß-glucuronide in dimethylformamide) was added. After an incubation of 16 h at 37 °C the appearance of a blue colour was indicative of GUS-activity. Chlorophyll was removed from stained tissues with ethanol extraction and transformed tissues were observed using a dissecting microscope. Unless otherwise mentioned, the transformation experiments were done

with 24 explants. Explants with more than 50 spots were counted as 50. The transformation susceptibility was determined as the mean number of GUS-positive spots per explant or as the percentage of explants with one or more spots.

Results

Ri roots

Carnation leaf explants were derived from *in vitro*-grown plants (CPRO 89074), infected with LBA9402(p35SGUSint) and incubated on hormone free medium. Highly branching, fast growing roots developed within 3 weeks, while under these conditions non-infected explants did not produce adventitious roots. The Ri roots were able to grow autonomously on MS medium without hormones, showing that the roots contained and expressed *Ri* genes.

Monitoring GUS expression in time

Leaf explants were examined for GUS activity during two weeks after infection. Two days after infection, GUS-positive spots were not yet detected, but after three days, the first spots were visible at the wound surfaces showing that the T-DNA of the binary plasmid was transferred and the *gus* gene under control of the CaMV 35S promoter correctly expressed. The number of spots increased in time reaching a maximum around day nine, with a mean number of 1.9 ± 0.7 (Fig. 1). At that time, 42 % of the explants showed GUS-expression. After 13 days, the number of blue spots had declined to a level of 0.21 ± 0.1 spots. At that time, 17 % of the explants showed GUS-expression. This decrease was probably due to the decline of transient expression. The first GUS-positive root (Fig. 2A) appeared after 13 days.

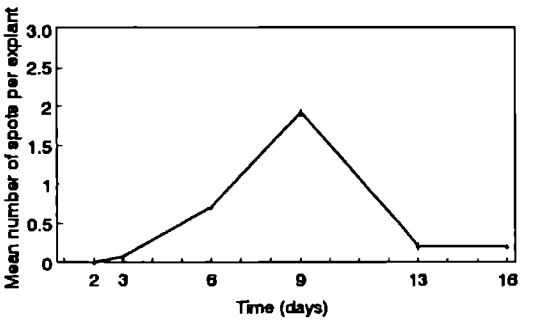

Figure 1. Development of *gus* expression in leaf explants derived from *in vitro*-grown plants (CPRO 89074) upon transformation with LBA9402(p35SGUSint). Values are the mean number of blue spot per explant in 24 observations. The s.e. is indicated by dotted lines.

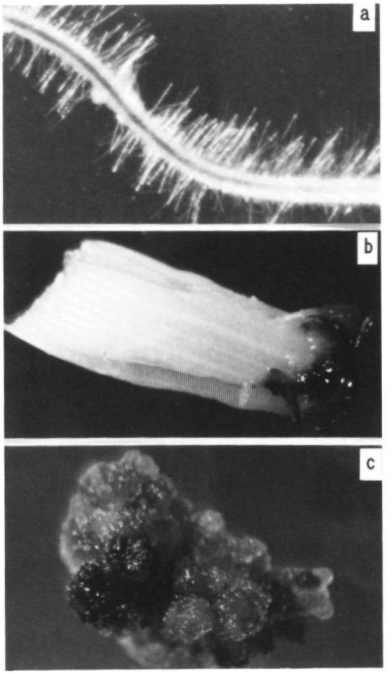

Figure 2. Presence of blue staining in carnation tissue. (A) GUS-positive root obtained 3 weeks after infection of leaf explants derived from *in vitro*-grown plants (CPRO 89074) with LBA9402(p35SGUSint). (B) Presence of blue staining in leaf explants derived from greenhouse-grown plant (CPRO 89100), 14 days after infection with LBA9402(p35SGUSint). (C) Presence of blue staining in transformed callus (CPRO clone 89086) developed after *A.rhizogenes* infection. Callus was derived from leaf explants derived from greenhouse-grown plants, and cultured on selection medium for six months.

Effect of leaf age

For regeneration of carnation leaf explants, the position of leaves on the plant appeared to be important. The youngest leaves just below the apical meristem gave the best shoot regeneration capacity (Van Altvorst *et al.*, 1992). To determine if susceptibility for *Agrobacterium* transformation was dependent on leaf age, the upper six leaf pairs from *in vitro*-grown plants (CPRO clone 89098) were inoculated with LBA9402(p35SGUSint) and after 14 days the number of GUS-positive spots was counted. As shown in Fig. 3, the youngest leaves showed the highest response. The same phenomenon was observed for a Sim carnation (CPRO 89117) and a spray carnation (CPRO 89077) (data not shown).

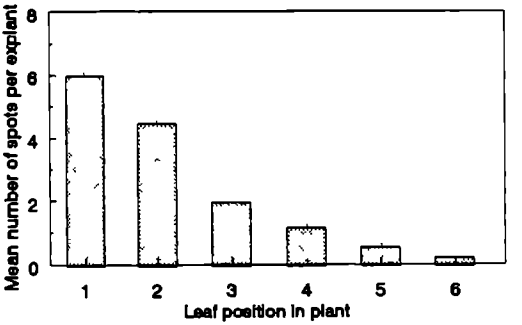

Figure 3. Effect of leaf age on the mean number of spots in CPRO clone 89098. Leaf explants were isolated from *in vitro*-grown plants and infected with LBA9402 (p35SGUSint). Each value is the mean ± s.e. of 24 explants.

Effect of leaf origin

To test the effect of plant culture conditions on susceptibility for transformation, leaf explants from six cultivars were excised from *in vitro*- and greenhouse-grown plants. After 14 days, 24 explants were tested for GUS-activity, and 24 explants were subcultured on medium without auxin: MS-30 with 0.9 mg/l BA. Fig. 4 shows the number of GUS positive spots after 14 days and the amount of callus and root formation after 8 weeks. The results clearly show that in all cultivars the greenhouse leaves were more susceptible for *A.rhizogenes*; they showed a higher number of GUS positive spots, and besides, the amount of callus and root production was larger. In some explants, large areas of the explant stained blue, making it difficult to estimate the number of blue spots accurately (Fig. 2B). All cultivars produced adventitious roots, except cultivar 89086 which showed abundant callus formation. Green calli from this clone and white calli from clone 89100 were further subcultured. All callus lines were GUS-positive and some of them produced small hairy roots, but none of the calli spontaneously produced adventitious shoots. In an attempt to obtain transformed shoots, 36 independently obtained calli from CPRO clone 89100 and 18 independently obtained calli of CPRO clone 89086 were subcultured for eight weeks on solid media with various concentrations of zeatin (0, 0.1, 1, 10 mg/l) with or without 0.3 mg/l NAA. On hormone free medium, some transgenic

calli of CPRO 89086 produced roots, and some calli of CPRO 89100 were able to grow autonomously (Fig. 5). Under these conditions non-transformed calli always failed to grow (data not shown). Although differences in growth rate between individual calli were observed, none of them produced adventitious shoots. Six months after callus initiation, the calli still showed blue staining (Fig. 2C), indicating that the *gus* gene was stably integrated and expressed.

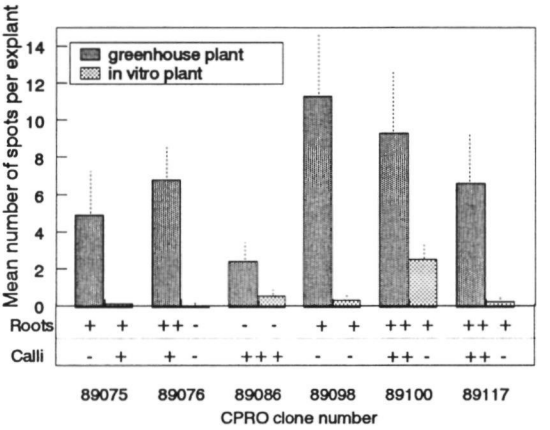

Figure 4. Effect of leaf explant origin (*in vitro*- versus greenhouse-grown plant) on the mean number of GUS-positive spots. Each value is a mean ± s.e. of 24 explants. The amount of callus and root growth was scored after 8 weeks by using an arbitrary scale from - (no calli/roots) to + (minor development of calli/roots) and + + (large number of calli/roots).

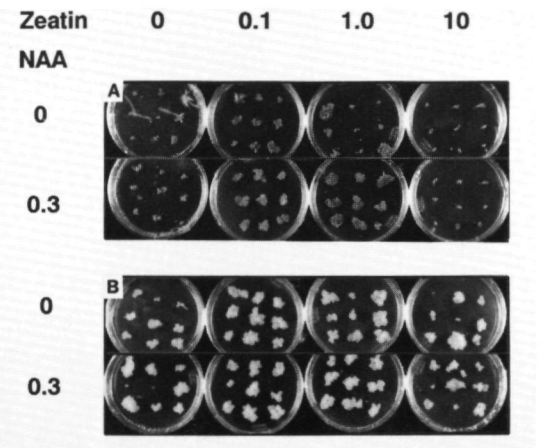

Figure 5. Growth of 9 independently obtained calli from CPRO 89086 (panel A) and from 89100 (panel B) on various concentrations (mg/l) of zeatin and NAA. Calli were obtained from greenhouse explants after infection with LBA9402(p35SGUSint) and subcultured for 8 weeks on the different regeneration media.

Discussion

Infection of carnation leaf explants with *A.rhizogenes* leads to root formation. The phenotype of the hairy roots and their autonomous growth on hormone free selection medium reflect the expression of Ri-genes. Genetic transformation of kanamycin resistant callus derived from such roots was confirmed by histochemical assays.

Cultivar differences in the susceptibility to infection with *A.rhizogenes* were detected. The difference in response to infection between leaves from greenhouse-grown and *in vitro*-grown plants was very remarkable. Leaf explants from greenhouse-grown plants showed a higher response to *A.rhizogenes* compared to leaf explants from *in vitro*-grown plants. Such a difference between *in vivo* and *in vitro* grown plants from the same genotype was also reported for pea upon *A.tumefaciens* infection (Hobbs *et al.*, 1989). It is likely to assume that anatomical and physiological modifications of the epicuticular wax, stomata and epidermal cells can influence the relationship between the carnation plant and the *Agrobacterium* strain.

Sometimes no correlation was observed between the number of GUS-positive spots and the amount of callus or root production. It is not likely to assume that this originated from differences in efficiency of T-DNA transfer between the *gus* gene and the Ri genes, because co-transfer frequencies in other binary vector systems were always recorded over 60 % (Hamill *et al.*, 1987; Sukhapinda *et al.*, 1987; Ottaviani and Hänisch ten Cate, 1991). Moreover, the development of callus and roots (both induction and growth) is not only dependent on the number of transformation events, but also on the hormonal status in the plant cell. Hormone concentration and hormone sensitivity will be changed by introduction of TL-DNA and TR-DNA (Gelvin, 1990).

In carnation transformation, the age of the leaf is important; the cells of the youngest leaves were the most susceptible for *A.rhizogenes*. We have previously shown (Van Altvorst *et al.*, 1992) that the youngest leaf base explants contained cells with a high regeneration potential, suggesting that the same cells were sensitive for regeneration and for *A.rhizogenes*-mediated transformation. The integration of foreign DNA might be facilitated during DNA replication in dividing cells. A similar effect of explant age on transformation frequency has also been observed for tobacco pith tissue with *A.rhizogenes* (Chriqui *et al.*, 1988), and for carnation with *A.tumefaciens* (see Chapter 6 of this thesis).

The main advantage of using *A.rhizogenes* instead of a derivative of *A.tumefaciens* is that the distinctive phenotype of transformed roots in culture is an excellent marker for transformation. Besides, the *A.rhizogenes* strain LBA9402 was more efficient in transferring T-DNA to plant cells than *A.tumefaciens* strains. A disadvantage of the *A.rhizogenes* system is that regeneration, if possible, leads to aberrant plants. Tepfer (1990) summarized the results of 37 species, in which transformed plants were obtained from *A.rhizogenes* induced hairy roots or callus. Only in tobacco and morning glory, shoot formation was achieved spontaneously (Tepfer, 1984; Brillanceau *et al.*, 1989), whereas in other species, regeneration occurred through hormonal induction via callus (Damiani and Arcioni, 1991) or via somatic embryogenesis (Tepfer, 1984; Sukhapinda *et al.*, 1987). However, the results presented in this chapter show that regeneration of transformed Ri carnation callus was not successful with combinations of zeatin and NAA. Shoot regeneration of Ri transformed cells might be hampered by auxin produced by *IAA* genes, as suggested by Birot *et al.* (1987). We therefore set out to develop a transformation system for carnation with a derivated *A.tumefaciens* strain, in spite of the potentials of the *A.rhizogenes* system.

Acknowledgements

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CHAPTER 6

TRANSGENIC CARNATIONS (*DIANTHUS CARYOPHYLLUS* L.) OBTAINED BY *AGROBACTERIUM TUMEFACIENS* MEDIATED TRANSFORMATION OF LEAF EXPLANTS

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(submitted)

Summary

For the development of an *Agrobacterium*-mediated transformation procedure of carnation (*Dianthus caryophyllus* L.), an intron containing ß-glucuronidase (*gus*) gene was used to monitor the frequency of transformation events soon after infection of leaf explants. The efficiency of gene transfer was dependent on the carnation genotype, explant age and cocultivation time. Leaf explants from the youngest leaves showed the highest number of GUS-positive spots. After selection on a kanamycin-containing medium transgenic shoots were generated among a relatively high number of untransformed shoots. The selection procedure was modified to improve the contact between explant and medium. This improved the selection and decreased the number of escapes. Kanamycin resistant and GUS-positive plants were obtained from five cultivars after infection of leaf explants with the supervirulent *A.tumefaciens* strain AGLO. A higher transformation frequency was observed with the binary vector pCGN7001 than with the p35SGUSint vector. Integration of the genes into the carnation genome was demonstrated by Southern blot hybridization. The number of T-DNA insertions varied between independent transformants from one to eight. Transformants were morphologically identical to untransformed plants. Segregation of the genes occurred in a Mendelian fashion.

Introduction

Lu *et al.* (1991) reported a transformation procedure for carnation, using stem explants as starting plant material. In this article we describe an efficient transformation procedure for carnation using leaf explants. As part of our research program to develop a gene transfer system in carnation, we have developed an efficient regeneration procedure starting from leaf base explants (Van Altvorst *et al.*, 1992). The efficiency of adventitious shoot formation from leaf explants of carnation has been further improved by some modifications in the explant isolation method (Van Altvorst *et al.*, 1994). In the present research this regeneration system was used for *Agrobacterium*-mediated transformation. Leaf base explants were cocultivated with the supervirulent strain AGLO (Lazo *et al.*, 1991), possessing the binary vector p35SGUSint (Vancanneyt *et al.*, 1990) or pCGN7001 (Comai *et al.*, 1990). These binary plasmids contain the

nptII (neomycin phosphotransferase II) gene to provide kanamycin resistance as a selectable marker, and the reporter gene ß-glucuronidase (*gus*) (Jefferson *et al.*, 1987).

The p35SGUSint plasmid contained a *gus* gene with an intron derived from potato gene LS1 (Vancanneyt *et al.*, 1990). This feature together with the high sensitivity of the staining procedure allows the detection of single transformation events soon after *Agrobacterium* infection without interference of bacteria which are still present. In order to analyze the effect of various factors which might influence T-DNA transfer, GUS expression was measured 14 days after inoculation. A number of conditions might influence the frequency of transformation events. Lu *et al.* (1991) used an extended cocultivation period of 5 days. They suggested that the cocultivation period should be long enough to allow induction, chemotaxis, attachment and gene transfer. Preliminary experiments with carnation leaf explants also showed that an elongation of the cocultivation period from 2 to 5 days enhanced the number of GUS-positive spots. In addition, Lu *et al.* (1991) used a high concentration of auxin in the cocultivation medium. However, high auxin concentrations result in proliferation of callus, even if only applied during cocultivation. It is well-documented that tissues which are cultured as callus, for even a relatively short period of time, may regenerate into plants which are variable in their morphology, physiology and karyotype, a phenomenon termed somaclonal variation (Karp and Bright, 1985). To limit the amount of callus formation, we have used a shoot regeneration medium with a very low concentration of auxin.

There is evidence that the transformation frequency depends upon the type of tissue (its age or location) used for inoculation, although the physiological basis for this is far from clear (Van Wordragen and Dons, 1992). Previous experiments showed that T-DNA transfer to carnation by *A.rhizogenes* was dependent on explant age (Chapter 5). There was a gradient for *A.rhizogenes* susceptibility, the youngest leaf explants showed the highest number of GUS-positive spots. Besides, greenhouse leaf explants were more susceptible for DNA delivery compared to leaf explants derived from *in vitro*-grown plants. Such an effect of explant type was also observed in preliminary experiments with *A.tumefaciens*. Transformation experiments described in this article, were therefore carried out with leaf explants from greenhouse-grown plants.

Material and methods

Plant materials

Thirty cultivars were used representing the various carnation (*Dianthus caryophyllus* L.) types and were indicated with CPRO clone numbers. Plants, obtained as rooted cuttings from commercial sources, were cultured in a conditioned culture room (about 70 % RH, 16 hours 18 °C in light and 8 hours 16 °C in dark). Nodal cuttings were surface sterilized, first 5 seconds in 70 % (v/v) ethanol, then for 20 min in a 2% (v/v) solution of sodium hypochlorite with 0.01% (v/v) Tween-20 and subsequently rinsed three times for 10 min in sterile water. Sterile cuttings of tobacco (*Nicotiana tabacum* 'Petit Havana SR1') were grown on MS medium with 2 % sucrose and without hormones. Four weeks old plantlets were used in transformation experiments.

Culture media and culture conditions

Leaf explants were isolated and regenerated according to Van Altvorst *et al.* (1992), using a modified shoot regeneration medium (SRM): MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose (MS-30), 0.5 g/l trypton, 1 mg/l 6-benzyladenine (BA), 0.1 mg/l α-napthaleneacetic acid (NAA) and 0.8% (w/v) purified agar (Oxoid). The cocultivation medium consisted of SRM with 100 μM acetosyringone. The selection medium (SM) consisted of SRM supplemented with 200 mg/l carbenicillin and 300 mg/l vancomycin to inhibit bacteria growth, and 100 mg/l kanamycin. The rooting selection medium (RSM) contained MS-30 supplemented with 0.1 mg/l NAA, 0.1 mg/l GA_3, 200 mg/l carbenicillin, 300 mg/l vancomycin and 200 mg/l kanamycin, and was solidified with 0.4% (w/v) Gelrite (Phytagel, Sigma). In preliminary experiments, gelrite-solidified media showed a lower effective selection with kanamycin. Therefore, the kanamycin concentration was enhanced to 200 mg/l. All media were adjusted to pH 5 8 before autoclaving. Antibiotics and acetosyringone were added after autoclaving. Culture conditions were 25°C and a photoperiod of 16 h with cool white light (Philips TL 85 fluorescent lamps, 36 μmol/m^{2}s).

Agrobacterium strains and plasmids

Both the octopine strain C58C1(pMP90) (Koncz and Schell, 1986) and the agropine strain AGLO (Lazo *et al.*, 1991) were used in transformation experiments. The strain AGLO was derived from A281, a supervirulent strain with a broad host range (Hood *et al.*, 1986). The binary vector p35SGUSint (Vancanneyt *et al.*, 1990) was used in the initial experiments to monitor GUS expression 14 days after infection. This vector is a derivative of pBIN19 and contained both a *gus* gene, driven by a cauliflower mosaic virus (CaMV35S) promoter, and a *nptII* gene, driven by the nopaline synthase (*nos*) promoter (RB...nos-*nptII*...35S-*gus-int* ..LB). The vector pCGN7001 (Comai *et al.*, 1990) contained both a *nptII* gene, driven by the CaMV35S-promoter, and a *gus* gene, driven by the mas5'-promoter (RB...mas5'-*gus*...35S-*nptII*...LB).

A.tumefaciens strains were cultured overnight at 28°C in liquid Luria Broth (LB, 1.0 % trypton, 0.5 % yeast extract, 0.5 % NaCl, 0.1 % glucose) medium supplemented with 100 μM acetosyringone, 100 mg/l rifampicin, 25 mg/l gentamycin and 50 mg/l kanamycin. Before inoculation the O D. at 550 nm was adjusted to 0.1 with liquid cocultivation medium.

Transformation method

Method A. Cuttings were harvested from greenhouse-grown plants and leaf explants were removed from the stem according to the regeneration protocol for leaf base explants (Van Altvorst *et al.*, 1992, 1994). Leaves from the first three nodes (position 1, 2 and 3) were used and explants with a length of about 20 mm were incubated for 5 min with *A.tumefaciens*. Per petri dish, 6 explants were incubated on cocultivation medium for 5 days. Every 3 weeks, explants were transferred to fresh SM. Green shoots of more than 5 mm length were removed from the explants and transferred to RSM. Control explants were incubated with bacteria without the binary vector. As a positive control, tobacco leaf explants were transformed according to the leaf-disk transformation method reported by Horsch *et al.* (1985).

Method B was carried out as described under method A, with the following modifications. Each time the explants were transferred (after 5 days, after 3 and 6 weeks), the length of the leaf explant was reduced to 5 mm. After 3 and 6 weeks, the base of the explant was divided into 2 or 3 pieces.
Method C was carried out as described under method B, with the following modification. After 5 days, the leaf explants were divided into two pieces.

Histochemical GUS assay and microscopical analysis
Plant tissue was assayed for ß-glucuronidase activity (GUS) according to Jefferson *et al.* (1987) using a modified extraction buffer containing: 100 mM sodium phosphate (pH 7.5), 10 mM Na_2-EDTA, 0.025 % thimerosal, 0.1 % Triton X-100. To 1 ml of this buffer 10 µl ironcyanide solution (21.1 mg/ml potassium ferricyanide and 16.5 mg/ml potassium ferrocyanide in H_2O) and 25 µl X-Gluc solution (20 mg ml^{-1} 5-bromo 4-chloro 3-indolyl ß-glucuronide in dimethylformamide) were added. Tissues were incubated in staining solution at 37 °C overnight (14-16 h) and subsequently chlorophyll was removed from the tissues by ethanol extraction. Explants were analyzed for transformed tissue (or GUS positive spots) using a binocular. To analyze the development of putative transformed tissue, explants were assayed for GUS expression at days 6, 12, and 18. After staining, explants were fixed in 2 % glutaraldehyde in 20 mM phosphate buffer (pH 7.2) supplemented with 135 mM NaCl for 1 h. After rinsing three times in phosphate buffer the explants were dehydrated gradually in a series of 50-100 % ethanol and fixed in a series of 25-100 % Technovit A (Kulzer). Tissues were embedded in Technovit 7100 according to the manufacturer's instructions. For light microscopy 8 µm serial transverse sections were cut using a disposable microtome blade (histoknife H, Kulzer) on a Jung microtome. Sections were mounted on glass slides. Air-dried sections were enclosed in euparal (Schmidt GmbH + Co) and examined using a Zeiss Axioskope microscope.

Data analysis
Agrobacterium susceptibility was determined as the mean number of GUS-positive spots per explant or as percentage of explants with one or more spots. The transformation percentage was determined as number of explants producing kanamycin resistant shoots divided by the number of infected leaves.
The data of the genotype experiment have been analyzed according to a generalized linear mixed model according to Jansen and Hoekstra (1993). Calculations were carried out using the computer program Genstat (Genstat 5 Committee, 1988).

Molecular analysis
Genomic DNA was isolated from leaves according to a procedure described by Van der Beek *et al.* (1992). Integration of the *gus* and *nptII* gene was demonstrated by Southern blot analysis. The DNA samples were digested with HindIII or EcoRI, separated on a 0.8 % w/v agarose gel, and blotted onto 'Hybond' nylon membrane. A 2.6 kb PstI fragment, containing the *gus* gene, and a 1.9 kb PstI fragment, containing the *nptII* gene, were excised from p35SGUSint and used as DNA probes. After hybridization with random primed [^{32}P]-labelled fragments (Feinberg and Vogelstein, 1983), the filter was washed and exposed to X-ray film at -80°C using intensifying screens.

Results

Susceptibility of carnation for *A.tumefaciens* transformation

In preliminary experiments thirty cultivars of carnation, belonging to various carnation types, were tested for their susceptibility for *A.tumefaciens* infection. From each cultivar, 12 leaf explants from greenhouse-grown plants were

cocultivated with C58C1(pMP90,p35SGUSint) and GUS staining was performed after 14 days. Nine cultivars clearly showed GUS positive spots with transformation frequencies ranging from 4 up to 50 %. All these cultivars have also been tested for their capacity to form adventitious shoots (data not shown). Based on regeneration and preliminary transformation results, a selection was made of five genotypes (viz CPRO clone number 89073, 89074, 89077, 89086 and 89127), which were also representing the various carnation types (Table 1). Leaves from these genotypes were subsequently infected with C58C1(pMP90,p35SGUSint) in 14 independent experiments, all carried out according to transformation method A. Within one cultivar, a large variation was observed in the percentage of GUS-positive leaf explants. In addition, between each pair of genotypes percentages varied from one experiment to another. Statistical analysis of the results indicated that genotype 89086 was less susceptible for *A.tumefaciens* than the other four genotypes (Table 1).

Table 1. Differences in susceptibility of various cultivars to *A.tumefaciens* strain C58C1(pMP90, p35SGUSint).

Cultivar (CPRO clone)	Carnation type	Total number of explants	Percentage of GUS-positive explants (± s.e.)
89073	spray	330	17.3 ± 2.2
89074	Mediterranean	372	16.8 ± 2.7
89077	spray	342	14.4 ± 2.0
89086	Diantini	300	4.7 ± 1.0
89127	Sim	372	15.8 ± 1.9

Results were obtained from 14 identical experiments, carried out according to method A.

Development of putative transformed tissue in leaf base explants

Histological analysis of AGLO(p35SGUSint) infected leaf explants (CPRO clone 89127) was performed to investigate the development of putative transformed tissue. After 6 days, cells expressing GUS were most frequently observed in the parenchymal cells of the cut surface (as shown in Fig. 1a). It is not likely that this region will produce transgenic shoots, since newly formed meristems developed directly from the subepidermal cells at the base of the explant (data not shown). Cells expressing GUS were also observed in the subepidermal cells in which promeristem initiation occurred. After 12 days, GUS-positive spots sometimes consisted of two cells (Fig. 1b) and organized masses of transgenic

cells were observed after 18 days (Fig. 1c). After 4 weeks, GUS-positive spots were still present as undivided cells or as clusters of about 20 cells. Also, transgenic primordia, strongly expressing GUS, were visible at the base of the explant (Fig. 1d). After 8 weeks, about a quarter of the GUS-positive spots consisted of more than 30 cells. At this time, explants without adventitious shoot formation were completely necrotic.

Transgenic plants

A large transformation experiment was carried out with CPRO clone 89127 using the supervirulent derivative AGLO harbouring the p35SGUSint plasmid. Plant material from greenhouse grown plants was harvested every two weeks over a long period (42 weeks), stored at 2 °C, and transformation was carried out using leaves at position 1, 2 and 3. Table 2 shows the result of the GUS analysis 14 days after transformation and clearly demonstrates that the youngest leaf (position 1) was very susceptible for transformation. All explants were selected on kanamycin containing media according to protocol A. The number of green shoots developed after 6 weeks dependent on the leaf position (Table 2). The youngest leaves showed the highest number of green shoots (on average 2.7 shoots per 12 explants), while leaves from position 3 showed the lowest number of shoots (meanly 0.9 shoots per 12 explants). The total number of shoots obtained from position 1 was 680, and two of them were able to root on a medium with 200 mg/l kanamycin. Moreover, both plants exhibited an intense blue staining (Fig. 1e).

Table 2. Effect of leaf age on regeneration and transformation of explants from CPRO clone 89127.

	Mean number of GUS-positive spots	Mean number of green shoots
position 1	45 ± 6	2.7 ± 0.8
position 2	19 ± 3	1.9 ± 0.5
position 3	2 ± 1	0.9 ± 0.2

Cuttings were harvested every two weeks during 42 weeks and leaves were inoculated simultaneously with AGLO(p35SGUSint).
The mean number of GUS-positive spots in 6 leaves was determined after 14 days. Explants with more than 20 spots were counted for 20.
The mean number of green shoots which were developed on 12 leaves was determined after 6 weeks.

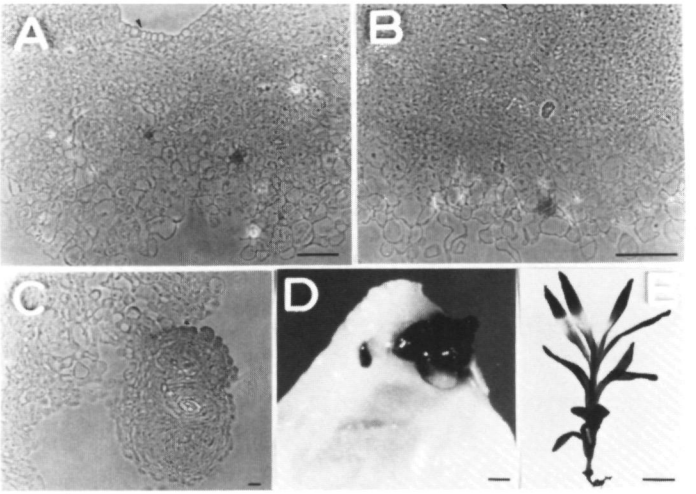

Figure 1. Analysis of cells expressing GUS in regenerating leaf explants. (A) After 6 days, cells expressing GUS were most frequently observed in the parenchymal cells of the cut surface. Arrow indicates upper epidermal cell layer. Bar = 100 μm. (B) After 12 days, a GUS-positive cell consisted of two cells. Bar = 100 μm. (C) An organized mass of transgenic cells was observed after 18 days. Bar = 10 μm. (D) A transgenic primordium was visible at the base of the explant after 4 weeks. Bar = 100 μm. (E) Transgenic carnation plant (89127-2) showing intense blue GUS-expression. Bar = 1 cm.

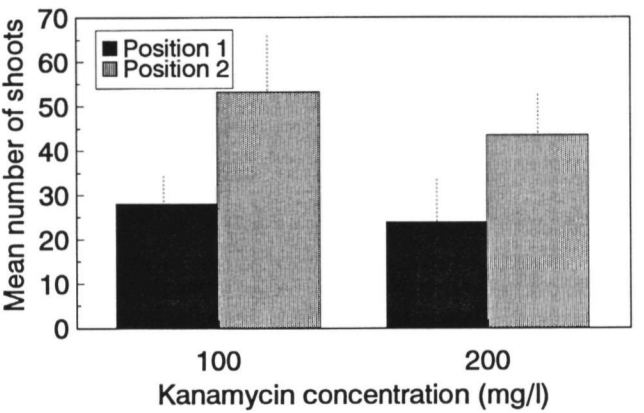

Figure 2. Effect of kanamycin concentration on the selection of carnation leaf explants (CPRO clone 89127). The results were obtained from one experiment carried out according to method A with cuttings from four harvest data. Per harvest date and per position, 108 leaf explants were infected with AGLO(p35SGUSint). The bars represent the total number of regenerants after 3 weeks based on 108 explants.

Improvement of the selection

Since the number of transgenic shoots among the regenerants was relatively low, it was assumed that improvement of the selection on kanamycin containing media might decrease the number of escapes. Because it was evident that very young leaves were most responsive both for shoot regeneration as well as for transformation, only leaf explants from position one and two were used in these experiments. Two parameters were changed to improve the selection on kanamycin.

(1) Concentration of kanamycin. Leaf explants were incubated on SM with a higher concentration of kanamycin: 200 mg/l. Figure 2 shows the results after 3 weeks. The number of regenerants obtained after 3 and 6 weeks was not decreased compared to control explants, which were incubated on SM with 100 mg/l. So, a higher concentration of kanamycin did not decrease the number of escapes.

(2) Modifications in the selection procedure. To improve the contact between the regenerating tissues and the kanamycin containing medium, the leaf explants were sectioned during subculture. As described in material and methods, this resulted in method B and C (schematically shown in Fig. 3). The modifications described in method B and C did not influence the shoot regeneration of control explants (data not shown). Large numbers of leaf explants from Sim type carnation plants (CPRO 89100, 89117, 89127 and 89132) were inoculated with either AGLO(p35SGUSint) or with AGLO(pCGN7001) and selected according to the three procedures. Table 3 summarizes the results obtained. Although the number of regenerants decreased considerably (method A 19-60; method B 3-23 and method C 4-8 regenerants per 100 leaves), the number of transgenic plants obtained was low in all procedures, resulting in transformation frequencies up to 1.5 %. The transformation percentage was dependent on the plasmid used. For AGLO(p35SGUSint), this percentage varied from 0.1 to 0.4 %, while for AGLO(pCGN7001), percentages were obtained from 0.4 to 1.5 %. The developed transformation procedure appeared to be applicable for the four tested Sim type carnations (presented in table 3), and besides, the procedure was successfully applied for a spray carnation (CPRO clone 89074). So far, 19 transgenic plants were obtained. Except two, all transgenic plants were derived from leaf position 1.

Table 3. Effect of transformation method used on the number of green shoots per 100 leaves and on the percentage of transgenic plants.

Method	Exp.	CPRO clone	Plasmid	Number of leaves	Total number of trans. plants	Number of shoots per 100 leaves	% of transgenic plants (on total number of leaves)	% of transgenic plants (on total number of shoots)
A	1	127	pGUS	504	2	19	0.4	2.1
	2	117	pCGN	240	1	38	0	0.3
		132	pCGN	240	0	60	0	0
	3	100	pCGN	204	3	59	1.5	2.5
B	4	127	pGUS	1080	1	6	0.1	1.5
	5	127	pGUS	84	0	10	0	0
			pCGN	84	1	8	1.2	14
	6	100	pGUS	228	0	3	0	0
			pCGN	198	2	9	1	12
		132	pGUS	90	0	19	0	0
			pCGN	90	1	23	1	5
C	7	127	pGUS	480	0	8	0	0
			pCGN	480	6	7	1.3	18
	8	117	pGUS	1128	1	4	0.1	2.5

Results are obtained from experiments with the youngest two leaf explants from Sim type carnations infected with AGLO(p35SGUSint) (=pGUS) or AGLO(pCGN7001) (=pCGN). Transformation methods were carried out as described under Material and methods.

Molecular analysis of transgenic plants

To analyze the presence of integrated genes, Southern blot analysis was performed on two kanamycin resistant and GUS positive plants from CPRO 89127 (89127-1, 89127-2), which were obtained after inoculation of leaf explants with AGLO(p35SGUSint). The copy number of the T-DNA insertion was determined from the number of hybridizing fragments that correspond to junctions between plant DNA and the T-DNA. Assuming random insertion of the T-DNA in the carnation genome, hybridization of *Eco*RI digested plant DNA with the *gus* probe (probe 1 in Fig. 4A) revealed bands that corresponded with individual *gus* genes. As shown in Fig. 4B seven or more *gus* genes were integrated in the genome of 89127-1 plants. *Hind*III digestion generated the expected internal fragment of 2.6 kb. However, four additional larger fragments were present. This indicates that only one of the two *Hind*III sites of the T-DNA fragment is present and suggests that these fragments were derived from incomplete insertions. When the *nptII* gene (probe 2) was used to probe *Eco*RI digested DNA, the same hybridization pattern was observed as that obtained with the *gus* probe. DNA from untransformed carnation plants did not show any DNA fragment hybridizing with the probes.

Because the Southern analysis of CPRO 89127-2 was less complex, this transgenic plant was used for further analysis and a detailed map of T-DNA insertions was constructed (see Fig. 4C). Using the *gus* gene as probe (probe 1), *Eco*RI digestion generated four hybridizing T-DNA fragments. Using the *nptII* gene as probe (probe 2), five *Eco*RI fragments hybridized, from which the largest fragment was very faint. This indicates that this fragment contained only a very small part of the *nptII* gene (fragment 1 in Fig. 4C) and thus was an incomplete T-DNA fragment. The smallest DNA fragment was smaller than the minimum expected size of 5.1 kb and did not hybridize with probe 1, indicating that this T-DNA fragment was also incomplete (fragment 5 in Fig. 4C). Using the *gus* gene as probe (probe 1), *Hind*III digestion generated the expected internal fragment of 2.6 kb, but also two larger fragments (Fig. 4B), indicating that in these fragments one of the two *Hind*III sites was not present (fragments 3 and 4 or fragments 1 and 4 in Fig. 4C). The combined results of the Southern analysis revealed that the genome of CPRO 89127-2 contained three (almost) complete T-DNA's (fragments 2, 3 and 4) and two partial T-DNA's (fragment 1 and 5). The present results of the Southern hybridization do not allow a discrimination between fragments 2, 3 and 4.

Apart from these two transgenic plants, the other 17 transgenic plants, which were obtained later, were also analysed. The number of T-DNA insertions in these plants varied between one and four (data not shown).

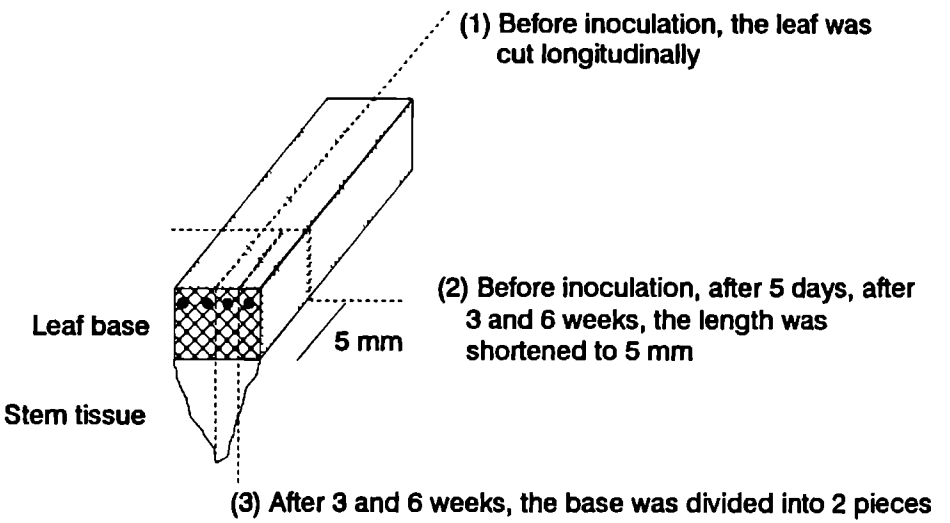

Figure 3. Schematic representation of transformation method B. After removing the leaves from the stem, the leaf is cut longitudinally (1) resulting in two explants from one leaf. After 5 days, cell divisions in the subepidermis of the leaf base result in the location of the first adventitious shoots (black circles). After 5 days, after 3 and 6 weeks, the length of the explant was shortened to 5 mm (2). After 3 and 6 weeks, the base of the explant was cut into 2 -3 smaller pieces (3). For transformation method C, the leaf base was also cut into 2 pieces after 5 days.

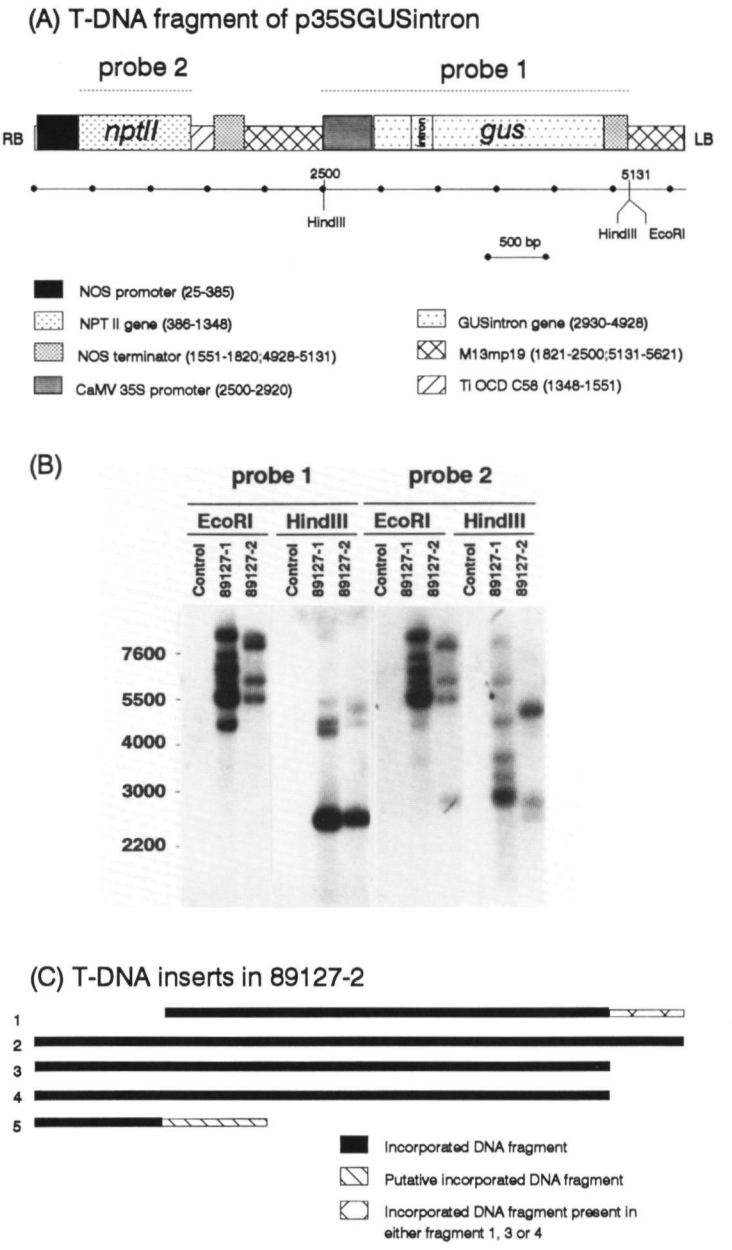

Figure 4. Southern blot analysis of transgenic carnation plants. (A) The 5.6 kb T-DNA region of vector p35SGUSint. (B) Southern blot analysis of DNA from CPRO 89127-1, CPRO 89127-2 and a non-transformed carnation plant (control). DNA was digested with *Eco*RI or *Hind*III and probed with the *gus* (probe 1) or the *nptII* (probe 2) gene. (C) The structure of plant T-DNA inserts deduced from Southern blot analysis of the transgenic plant CPRO 89127-2 (see text).

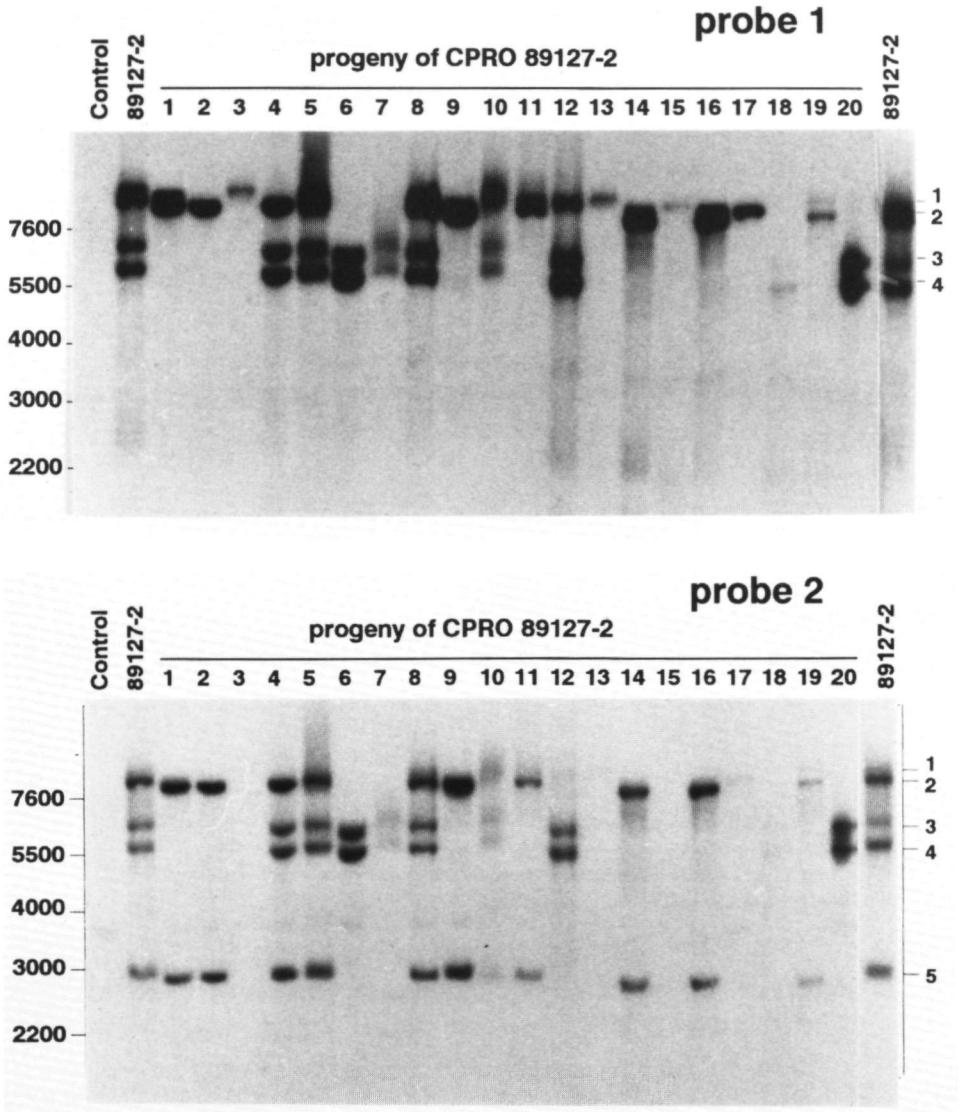

Figure 5. Southern blot analysis of DNA from CPRO 89127-2 and 20 GUS-positive progeny plants. DNA was digested with *Eco*RI and probed with (A) the *gus* gene (probe 1) or (B) the *nptII* gene (probe 2).

Segregation of the introduced genes

CPRO 89127-2 was used to study the segregation of the introduced genes in the progeny. CPRO 89127-2 flowers were fertilized with pollen derived from a non-transformed carnation plant ('Novada'). Seed of the primary transformant did not show any abnormalities in colour, size and germination efficiency compared to seed from the non-transformed control plant fertilized with 'Novada' pollen. One-hundred seedlings were germinated and after 4 weeks, one leaf from each plant was tested for GUS-activity. The segregation ratio was 89:11 (GUS^+:GUS^-) suggesting that CPRO 89127-2 might have active *gus* genes at three loci. From 20 GUS-positive seedlings, DNA was isolated, digested with *Eco*RI and the segregation of the T-DNA insertions was determined. The results of Southern blot hybridization are shown in Fig. 5. As described above, the primary transformant CPRO 89127-2 revealed four hybridizing T-DNA fragments (1,2,3,4) with the *gus* gene as probe (probe 1) and five fragments with the *nptII* gene as probe (probe 2). In all plants of the offspring at least one fragment was present in agreement with the positive GUS assay of these plants. Segregation analysis for each of the five fragments showed that approximately half of the progeny contained the fragment. This indicates that the transgenes segregated in a Mendelian fashion. Three groups of fragments were segregating independently. Fragments 3 and 4 and fragments 2 and 5 were cosegregating whereas fragment 1 segregated independently.

Discussion

This research has successfully demonstrated that *A.tumefaciens* can be used to obtain transgenic plants in at least five carnation cultivars. Southern blot techniques confirmed the transgenic nature of regenerated plants. The segregation ratio and Southern blot analysis indicated Mendelian inheritance of the foreign genes in the progeny of the transgenic carnation plant.

In this article, factors are described which influence the frequency of transformation events of carnation leaf explants and factors which influence selection and screening. The youngest leaves were the most susceptible for *A.tumefaciens* infection. This holds both for the number of transformation events (determined by the number of GUS-positive spots) and for the total number of transgenic plants (17 transgenic plants were derived from position 1, and 2 plants were derived from position 2). Sushan and Johnson (1955)

reported that in carnation, the youngest leaves mostly contained non-differentiated cells, which have a high division rate. An (1985) suggested that rapid division of cells is a critical factor for efficient transformation of tobacco. It is likely to assume that there is a correlation between cell division and optimal transformation.

Shoots harvested from the leaf base three weeks after transformation were never transgenic. This suggests that initially non-transgenic regenerants develop, whereas transformed and kanamycin resistant cells slowly divide in the presence of kanamycin and transgenic plants appear on the explants six or nine weeks after inoculation. Sometimes, chimeric GUS-positive callus was observed, which might result in chimeric plants. However we never found evidence for chimerism in the transgenic plants, indicating that the kanamycin concentration was sufficient to kill non-transformed cells.

In the carnation transformation experiments described, the number of false-positive plants which escaped the kanamycin selection, was high. Compared to tobacco leaf explants, carnation leaf explants were only slowly affected by kanamycin. We suggest that a slow penetration of kanamycin in carnation leaf explants might hamper the selection in an early stage. Indeed, improving the kanamycin penetration by some modifications in the selection procedure resulted in a much lower percentage of non-transformed shoots.

In our experiments, the transformation efficiency was depended on the binary plasmid used. The plasmid pCGN7001 showed higher transformation percentages compared to p35SGUSint. In pCGN7001 the *nptII* gene is controlled by the constitutive and non-inducible CaMV35S promoter (Odell *et al.*, 1985), while in p35SGUSint the *nptII* gene was driven by the nopaline synthase *nos*-promoter (Depicker *et al.*, 1982). In transgenic tobacco plants, the CaMV35S promoter resulted in approximately 20 to 30 fold higher levels of expression for the *nptII* gene than did the *nos*-promoter (Sanders *et al.*, 1987). It is likely to assume that the CaMV35S promoter also provides a higher level of expression in carnation leaf cells. Consequently, pCGN7001 transformed cells have a competitive advantage over p35SGUSint, resulting in a better outgrowth of transgenic cells. Moreover, the p35SGUSint plasmid contained a mutated *nptII* gene (Van Wordragen, pers. com.), while the pCGN7001 plasmid contained a non-mutated *nptII* gene (results not shown). The mutation in the *nptII* gene reduces the effectiveness of the kanamycin resistance (Yenofski *et al.* 1990), which subsequently also decreases the recovery of transgenic plants.

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APPENDIX CHAPTER 6: TRANSGENIC CARNATION PLANTS OBTAINED BY *A.TUMEFACIENS* MEDIATED TRANSFORMATION OF PETAL EXPLANTS

H.J.J. Koehorst, A.C. van Altvorst and J.J.M. Dons

Introduction

In addition to the successful transformation of leaf explants, we also have used petal explants of carnation for *A.tumefaciens*-mediated transformation. Adventitious shoot formation from carnation petal explants has been described by Kakehi (1979) and Gimelli *et al.* (1984). However, hyperhydricity (abnormally glassy, thick and translucent origin of the plants) and premature flowering of the shoots was a severe problem (Leshem, 1986, Van Altvorst *et al.*, 1992). There is growing evidence that cultured tissues in some way 'remember' where they originate from and this memory influences morphogenesis and genetic stability in culture (for a review, Meins, 1986). This phenomenon might explain the aberrant plant growth of petal regenerants resulting in early flowering.
Lu *et al.* (1991) used petal explants for *Agrobacterium*-mediated gene transfer. They obtained one GUS-positive shoot after infection of 200 petal explants of cv 'White Sim'. However, they did not show molecular evidence for the incorporation of genes in this plant, neither by PCR nor by Southern blot analysis. Besides, no information was presented on the phenotype of the transgenic plant and transfer of the shoot to the greenhouse. In this report, we also describe the introduction of genes into carnation petal explants. The two successful experiments showed some advantages and disadvantages of the use of petal explants.

Materials and methods

Isolation of petal explants from CPRO 89100 was carried out as described by Van Altvorst *et al.* (1992). Transformation of petal explants was carried out essentially as described for leaf explants. The standard regeneration medium of petal explants contained MS-30 with 1 mg/l 6-benzyladenine (BA) and 0.1 mg/l α-napthaleneacetic acid (NAA). In a first experiment, petal explants were cocultivated for 4 days on medium without cytokinin, but with 2 mg/l NAA (0-2). In a second experiment, petal explants were cocultivated for 2 days on medium without any hormones (0-0), with 2 mg/l NAA (0-2), 5 mg/l NAA (0-5) or with 1 mg/l BA and 0.1 mg/l NAA (1-0.1). Explants were selected on medium with 50 mg/l kanamycin (first experiment), or on 25 mg/l kanamycin (second experiment). After three weeks, explants were transferred to selection

medium with 100 mg/l kanamycin. Regenerants were obtained from the explants after 3, 6 and 9 weeks. Green and yellow-green shoots were transferred to rooting medium with 200 mg/l kanamycin and one leaf was tested for GUS-expression.

Results

In previous experiments, the regeneration capacity of petal explants was tested in a range of cultivars (Van Altvorst *et al.*, 1992). Fifty-five cultivars were tested and 37 of them showed regeneration, although most cultivars produced hyperhydric and early flowering regenerants (Fig. 1). These aberrant regenerants could not be transferred to the greenhouse. Three cultivars (CPRO 89086, 89100 and 89105) produced vegetative shoots which survived the transfer to the greenhouse. The transformation experiments were carried out with CPRO 89100. This cultivar was a Sim type carnation and was successfully used in leaf base transformation experiments. In preliminary transformation experiments, the regeneration percentage of non-infected petal explants of this cultivar was high, 81 ± 3 %.

In a first experiment, 30 petal explants were infected with AGL0(p35SGUSint) and 50 petal explants were infected with AGL0(pCGN7001). After six weeks, the mean number of shoots per explant was 0.87 ± 0.18. No differences were observed between the *A.tumefaciens* strains. Twelve weeks after infection, 70 plants were assayed for GUS activity and only two plants were positive. One GUS-positive plant (PT-1) had developed after infection with AGL0(p35SGUSint), the other (PT-2) after infection with AGL0(pCGN7001). Compared to control plants, regenerants obtained from infected petal explants were mostly hyperhydric, grew slowly and did not elongate (Fig. 2). As shown in Fig. 3, the GUS activity in PT-2 plants was mainly observed in older leaves. Vegetative shoots of PT-2, which were no longer hyperhydric, were transferred to the greenhouse. These plants survived the transfer to the greenhouse, but ceased growing and produced premature flowers.

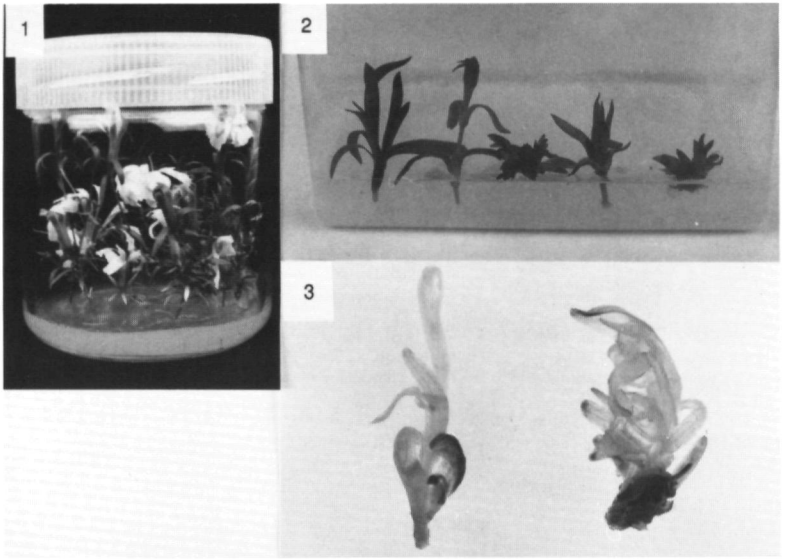


Figure 1. Premature flowering shoots developed on petal explants from CPRO 89105.
Figure 2. Transgenic shoots from CPRO 89100. From left to right, control plant 89100, PT-2, PT-3, PT-4, PT-1.
Figure 3. GUS-positive shoot (PT-2) generated after infection of petal explants with AGLO(pCGN7001).

In a second experiment, about 70 petal explants derived from CPRO 89100 were infected with AGL0(p35SGUSint) and AGL0(pCGN7001), and cocultivated on a range of hormone concentrations. Concerning the mean number of adventitious shoots per explant, no difference was observed between the cocultivation media (see Material and methods). A high number of shoots was obtained on petal explants cocultivated on hormone free medium, medium with only 2 mg/l NAA, and medium with 1 mg/l BA and 1 mg/l NAA, (Table 1). On medium with a high auxin concentration (0-5), almost no regeneration was observed. After 16 weeks, some plants were able to root on selection medium (indicated with 'R' in table 1), but only one of them was GUS-positive (PT-3). This plant was generated after infection of AGLO(pCGN7001) and after cocultivation on hormone free medium. In this experiment also another GUS-positive plant was obtained (PT-4). This plant was generated after infection with AGLO(p35SGUSint) and after cocultivation on 2 mg/l NAA. After 24 weeks, PT-4 rooted on selection medium. Both PT-3 and PT-4 were hyperhydric, formed short internodes, produced many axillary shoots and grew slowly.

Table 1. Effect of hormone concentrations (BA-NAA) in the cocultivation medium on the mean number of green or yellow-green shoots.

	AGLO(p35GUSint)				AGLO(pCGN7001)			
BA-NAA concentration (mg/l)	0-0	0-2	0-5	1-1	0-0	0-2	0-5	1-1
Number of infected petal explants	77	78	75	49	76	65	75	64
Mean number of shoots*								
- after 6 weeks	0.30	0.37	0.01	0.37	0.42	0.28	0.01	0.58
- after 10 weeks	0.12^{R}	0.17	0	0.06	0.22^{R}	0.18	0	0.19
- after 16 weeks	0.04	0.04	0	0.02^{R}	0.03^{R}	0.05	0	0.03^{R}

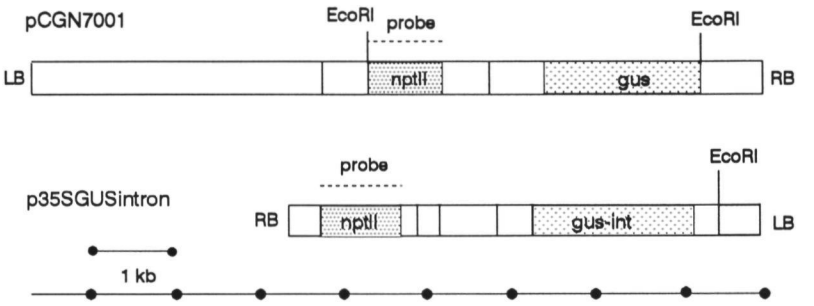
*Results show the mean number of shoots per explant 6, 10 and 16 weeks after infection of petal explants from CPRO 89100 with AGLO(p35SGUSint) or AGLO(pCGN7001).
Root formation of regenerated shoots was observed in treatments marked with 'R'.

To demonstrate the transgenic nature, molecular analysis of the four transformants was performed. DNA was isolated and PCR analysis showed the presence of the *gus* gene (data not shown). Southern blot analysis of inserted genes indicated the integration of the *nptII* gene in PT-2 and PT-3. These plants were obtained after infection with AGLO(pCGN7001). PT-4 and PT-1 were generated after infection with AGLO(p35SGUSint). Southern blot analysis indicated the integration of one *nptII* gene in PT-4 and two *nptII* genes in PT-1 (Fig. 4).

Discussion

This research has demonstrated that *A.tumefaciens* can be used to obtain transgenic plants from petal explants. Southern blot techniques confirmed the transgenic nature of the four transformed plants. Besides, the expression of the *gus* gene was verified in these plants by histochemical assays on selected shoots. In the described carnation transformation experiments, the number of false-positives was high and expression of *gus* was highly variable in and between transgenic plants. None of the transgenic plants showed a strong, consistent blue pigmentation. This variable expression of the *gus* gene might be due to the bad physiological conditions of these plants. There was no visual evidence, such as sectoring or tissue specific expression, for chimerism. In spite

of the potential of petal explants to obtain transgenic plants after *A.tumefaciens* infection, petal explants were not suitable for transformation due to the hyperhydricity of the regenerants and premature flowering.



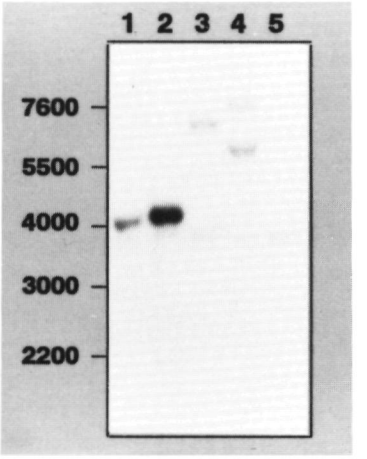


Figure 4. Southern blot analysis from four transgenic plants and a control plant.
PT-2 (lane 1) and PT-3 (lane 2) were obtained after infection with AGLO(pCGN7001), PT-4 (lane 3) and PT-1 (lane 4) were generated after infection with AGLO(p35SGUSint). DNA from PT-2, PT-3, PT-4, PT-1 and a non-transformed plant (lane 5) were digested with *Eco*RI and probed with the *nptII* gene. The 8.6 kb T-DNA region of pCGN7001 and the 5.6 kb T-DNA region of p35SGUSint are shown in the upper lines.

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CHAPTER 7

THE ROLE OF ETHYLENE IN THE SENESCENCE OF CARNATION FLOWERS, A REVIEW

A.C. van Altvorst and A.G. Bovy

(*Submitted*)

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1 Introduction

The regulation of petal senescence is of great interest to horticulturists in search of methods to improve the postharvest longevity of cut flowers. The senescence process is mediated by a series of highly coordinated physiological and biochemical changes, such as increased activity of hydrolytic enzymes, degradation of starch and chlorophyll, loss of cellular compartmentation, and a climacteric surge in respiration. These changes are associated with changes in gene expression and synthesis of proteins (Borochov and Woodson, 1989). In many plant species the phytohormone ethylene regulates flower senescence. In addition, ethylene is an important regulator of plant defense responses (for a review, see Boller, 1991), fruit ripening, leaf abscission, seed germination and many other processes in plant growth and development (reviewed by Reid, 1987).

Most of our knowledge concerning the process of ethylene-mediated flower senescence is derived from studies on the cut flower carnation (*Dianthus caryophyllus* L.). This review describes the progress made in this field over the past decade. It focusses on the (molecular) mechanisms underlying the regulation of ethylene biosynthesis and ethylene sensitivity. Furthermore, the possibility of genetic engineering aimed at extending the vase-life of carnation flowers will be discussed.

2 Flower senescence

The role of ethylene

The plant hormone ethylene is the smallest of all plant growth substances and one of the most pleiotropic effectors. Woltering and Van Doorn (1988) studied the role of ethylene in petal senescence of flowers of 93 species representing 23 families. Apart from the family of Caryophyllaceae (to which carnation belongs) also flowers from some other families (Campanulaceae, Malvaceae and most Orchidaceae) were sensitive to ethylene and showed wilting as the primary senescence symptom. In a number of plant species petals abscised in response to ethylene. On the contrary, in many monocot species petal wilting occurred without any apparent involvement of ethylene (Woltering and Van Doorn, 1988; Reid and Wu, 1992).

The senescence of carnation flowers is associated with a climacteric increase in the production of ethylene (Nichols, 1966; Maxie *et al.*, 1973). This ethylene production is autocatalytic, which means that exposure to ethylene stimulates ethylene biosynthesis (Woodson and Lawton, 1988). Ethylene serves to initiate and to regulate the processes that finally lead to programmed organ death. The early onset of ethylene synthesis during senescence of carnations and the retardation of senescence by inhibitors of ethylene biosynthesis indicate the importance of ethylene in this process. The ethylene biosynthetic pathway is highly controlled by both positive and negative feedback regulation, as well as by internal, environmental and stress factors (Abeles *et al.*, 1992).

Pollination

It is not surprising that flower longevity decreases after pollination. The advantages to the plant are twofold. Firstly, the growth of excessive pollen

tubes may result in competition for stylar food reserves which are required to nourish the growing pollen tubes. Secondly, the maintenance of elaborate floral structure must be a costly process, both in terms of respiratory energy used and the transpiration of water. If this can be minimized it must be to the plants advantage (Stead, 1992).

Pollination of carnation flowers results in irreversible wilting of petals within 1 to 2 days, whereas petals from unpollinated flowers senesce in 6 to 7 days (Nichols, 1977). Nichols showed that within 3 h after pollination the production of ethylene by the stigmas increased 10-fold. Only a small increase in ethylene production from the ovary and receptacle was detectable within this time period. Ethylene production by the petals started to increase after 8 h (Nichols *et al.*, 1983). The rapid increase in ethylene production upon pollination suggests the movement of a stimulus (a transmissible factor) for ethylene production from the stigma to the other flower organs. Both ethylene and 1-amino cyclopropane-1-carboxylic acid (ACC) may be responsible for this movement. Besides, there is evidence that short chain saturated fatty acids are involved (see also section 5).

3 Ethylene biosynthesis

The ethylene biosynthetic pathway

Biochemical aspects of ethylene biosynthesis have been reviewed extensively (Yang and Hoffman, 1984; Imaseki, 1991; Abeles *et al.*, 1992; Yang and Dong, 1993). In this paper we will briefly describe the ethylene biosynthetic pathway. The first step in the pathway is the conversion of methionine and ATP to S-adenosylmethionine (SAM), catalyzed by the enzyme SAM synthetase (Figure 1). SAM is converted by ACC synthase to 1-amino cyclopropane-1-carboxylic acid (ACC) and 5'-methylthioadenosine (MTA). MTA can be recycled to methionine, thus allowing high rates of ethylene production even if methionine concentrations are low. ACC is the immediate precursor of ethylene. Oxidation of ACC by the enzyme ACC oxidase (also known as the ethylene forming enzyme EFE) results in the production of ethylene, CO_2 and HCN. HCN is tought to be detoxified through conversion to ß-cyanoalanine by the enzyme ß-cyanoalanine synthase (Manning, 1986).

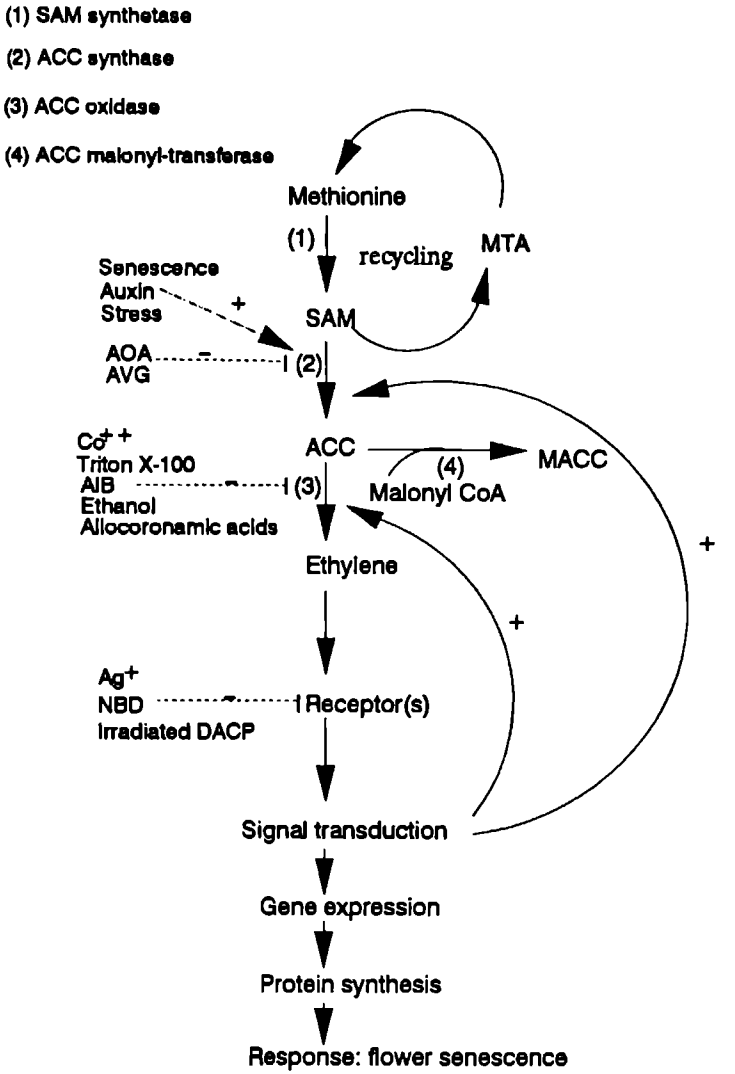

Figure 1. Schematic overview of ethylene biosynthesis, signal transduction and effects.

Abbreviations: ACC = 1-aminocyclopropane-1-carboxylic acid, AIB = α-aminoisobutyric acid, AOA = amino oxyacetic acid, AVG = aminoethoxyvinyl glycine, DACP = diazocyclopentadiene, EFE = ethylene forming enzyme, MACC = malonyl 1-aminocyclopropane-1-carboxylic acid, MTA = 5'-methylthioadenosine, NBD = 2,5 norbornadiene, SAM = S-

adenosyl-methionine, STS = silver thiosulphate

ACC

Ethylene biosynthesis can be regulated by conjugation of the ethylene precursor ACC. This conjugation is thought to be largely irreversible. The conjugated form of ACC has been identified as a malonyl conjugate (MACC). Young carnation petals have been shown to possess a more active system for the conjugation of ACC compared to senescing petals, indicating that this reaction may be used to limit the availability of free ACC as a substrate for ACC oxidase (Peiser, 1986). Isolated petals taken from young carnation flowers lasted longer than similar petals attached to the flower, suggesting that some factor outside the petals influenced the timing of petal senescence (Mor *et al.*, 1980). Hsieh and Sacalis (1986) showed that within 24 h after applying [^{14}C]ACC to the styles, 50% of the total radioactivity had migrated to other floral organs. They suggested that ACC was translocated from the gynoecium to the petals.

The spatial regulation of ethylene biosynthesis within carnation flower petals was investigated in more detail. When detached carnation petals, separated into upper and basal parts, were exposed to ethylene, autocatalytic ethylene production mainly occurred in the basal parts. Upper parts produced ethylene at a four times lower rate and contained lower levels of ACC synthase and ACC oxidase mRNAs (Drory *et al.*, 1993). Low concentrations of ethylene induced changes in enzyme activity in the upper petal parts. However, without the addition of ACC, ethylene did not lead to high levels of ethylene and visible wilting symptoms (Woltering *et al.*, 1991). Taken together the results indicate that ethylene production in the upper part of petals is largely the result of transport of ACC and ethylene from the basal tissue. Ethylene initially induces the expression of the ACC oxidase gene, which in turn leads to the production of ethylene from ACC (Overbeek and Woltering, 1990).

ACC synthase and ACC oxidase

In carnation, the increase in ethylene biosynthesis associated with petal senescence is accompanied by an increase in ACC synthase activity and ACC content (Peiser, 1986). This increase in ethylene production was associated with a dramatic increase in the abundance of the mRNAs encoding ACC synthase and ACC oxidase, while these mRNAs were undetectable in presenescent petals (Woodson *et al.*, 1992). Treatment of climacteric carnation petals with the ethylene response inhibitor 2,5-norbornadiene (NBD) resulted in inhibition of

ACC synthase and ACC oxidase activities, suggesting that continued synthesis of both enzymes is required (Woodson, 1991).

ACC synthase - The rate limiting step in the ethylene biosynthetic pathway is the conversion of SAM to ACC, catalyzed by ACC synthase (Woodson and Lawton, 1988). ACC synthase is most likely a cytoplasmic enzyme (reviewed by Kende, 1993). cDNA clones have been isolated from carnation, tomato, squash, *Petunia*, *Arabidopsis* and various other crops (reviewed by Van der Straeten and Van Montagu, 1991). To date, at least 5 different tomato ACC synthase cDNAs have been isolated, indicating that ACC synthase is encoded by a gene family (Rottmann *et al.*, 1991). The expression of ACC synthase genes is regulated by different stimuli, and different genes are responsible for ethylene production, for example during senescence or in response to wounding (Rottmann *et al.*, 1991; Nakajima *et al.*, 1990).
In carnation two ACC synthase genes have been identified, CARACC3 (Park *et al.*, 1992) and CARAS1 (Henskens *et al.*, 1993). It was shown that these two genes are expressed in a tissue-specific manner: CARACC3 mRNA was most abundant in the petals while CARAS1 mRNA was most abundant in the styles. Although a high ethylene production was also observed in ovaries, the level of both messengers was low in this organ. This might indicate the existence of a third ACC synthase gene that is expressed in the ovary.

ACC oxidase - The ACC oxidase enzyme is recalcitrant to purification, and consequently, the isolation of the corresponding gene(s) has been difficult. The first ACC oxidase cDNA clone, called pTOM13, was identified in tomato by using an antisense RNA strategy (Holdsworth *et al.*, 1987; see also section 7). To date 3 genes have been isolated from tomato (Holdsworth *et al.*, 1988; Köck *et al.*, 1991), indicating that like ACC synthase, ACC oxidase may also be encoded by a gene family. Based on sequence homology to the tomato p-TOM13 cDNA clone, Wang and Woodson (1991) reported the cloning of a putative ACC oxidase cDNA from carnation; pSR120. The level of the corresponding mRNA increased concomitant with the increase in ethylene production during senescence. The cellular localization of ACC oxidase is not clear yet. Biochemical studies indicated that ACC oxidase required intact membranes for its activity. However, based on the predicted amino-acid sequences of the three different tomato ACC oxidase genes, Hamilton *et al.* (1990) suggested that ACC oxidase is located in the cytosol, since it did not

show an obvious signal sequence. Until recently the activity of the enzyme could not be measured *in vitro* because once the tissue was homogenized, the ACC oxidase activity completely disappeared. As shown for melon and avocado ACC oxidases (Ververidis and John, 1991; Mc Garvey and Christoffersen, 1992), carnation petal ACC oxidase is a soluble enzyme (Neijenhuis *et al.*, 1994).

Inhibitors of ACC synthase and ACC oxidase

ACC synthase belongs to the group of enzymes that require pyridoxal phosphate as cofactor. Likewise, ACC synthase is sensitive to inhibitors of this type of enzymes such as aminooxyacetic acid (AOA), aminoethoxyvinylglycine (AVG) and its analogues (Baker *et al.*, 1977). Kirchner *et al.* (1993) recently described the effects of new derivates of AOA on the inhibition of ethylene biosynthesis. The activity of ACC oxidase can be blocked by Co^{2+} (Satoh and Esashi, 1983), Triton X-100 (Harkema *et al.*, 1987; Bichara and Van Staden, 1993), α-aminoisobutyic acid (AIB) (Satoh and Esashi, 1983) and allocoronamic acid derivates (Toshima *et al.*, 1993). Since inhibitors of ACC synthase and ACC oxidase only prevent ethylene biosynthesis, they offer little protection in ethylene-polluted environments.

Ethanol - Continuous treatment with 8 % ethanol doubled the vase life of 'White Sim' flowers (Wu *et al.*, 1992). During their eventual senescence, the petals of ethanol-treated flowers did not inroll. Very little ethylene was produced by ethanol-treated flowers, and the normal increase in ACC content and ACC oxidase activity was also suppressed. Ethanol treatment also decreased the sensitivity of the flowers to exogenous ethylene. Wu *et al.* (1992) suggested that in addition to inhibiting ethylene synthesis, ethanol interferes with ethylene action, but in some other way than by competing with ethylene for its binding site. Although ethanol is a useful additional tool to study the role of ethylene in flower senescence, it has no practical value because of the need to treat flowers continuously.

Effects of auxins, cytokinins and gibberellic acids

Several plant hormones have been shown to influence ethylene metabolism in carnation (reviewed by Cook and Van Staden, 1988). Auxins are thought to promote petal senescence through the stimulation of ACC-synthase activity (Nichols, 1971; Wulster *et al.*, 1982). The cytokinins kinetin, benzyladenine,

zeatin and dihydrozeatin have been shown to extend the longevity (Mor *et al.*, 1983; Van Staden *et al.*, 1987; Bossé and Van Staden, 1989), although the results were inconsistent. When very low concentrations of BA were supplied to the vase solution, senescence was even stimulated (Woodson and Brandt, 1991). It was suggested that this cytokinin-induced senescence is modified through an interaction with the gynoecium that leads to ACC accumulation and premature ethylene production in both the gynoecium and the petals.

Concerning gibberellic acids, it has been shown that GA_3 (giberrelic acid) delayed carnation senescence, when applied to young flowers. In all flower parts endogenous levels of ACC were reduced with GA_3 treatment. This was most pronounced in the petal bases (Saks and Van Staden, 1993a, 1993b). As already mentioned, petal bases are important regulatory sites for ethylene production, they may be involved in controlling the onset and degree of petal inrolling. An early decline in endogenous gibberellin levels in carnations is one of the correlative events associated with the termination of maturation and the onset of the senescence process (Saks and Van Staden, 1992a, 1992b).

4 Ethylene response

Ethylene sensitivity

The ethylene response is accompanied by an autocatalytic induction of several enzymes involved in the synthesis of ethylene. One possible mechanism to account for the induction of ethylene biosynthesis is a change in tissue responsiveness or sensitivity to ethylene. The sensitivity of floral petals to ethylene increases with age. Older petals start to senesce in response to lower concentrations of ethylene (Woodson and Lawton, 1988). This increase in responsiveness of carnation petals was not related to an increased ethylene binding capacity (Brown *et al.*, 1986). It was suggested that the increase in sensitivity may result from the synthesis of an ethylene sensitivity factor. Short chain saturated fatty acids are possible candidates for this factor, since pollination of carnation styles induced fatty acid synthesis and an increase in ethylene sensitivity. In addition, application of octanoic acid to the styles of unpollinated flowers resulted in an increase in ethylene sensitivity and a marked acceleration of petal senescence (Whitehead and Vasiljevic, 1993). The increase in ethylene sensitivity during the preclimacteric stage was associated with the accumulation

of short-chain saturated fatty acids (C_7 to C_{10}) produced in the petals during the early stages of senescence. These short-chain fatty acids might increase the ethylene binding affinity by altering certain membrane properties. Following pollination, it was shown that these fatty acids were produced in the style and transported to the corolla. The suppression of the ethylene response by treatment with STS was accompanied by a drastic suppression of fatty acid synthesis (Whitehead and Vasiljevic, 1993). We therefore suggest that the pollination-induced increase in ethylene sensitivity is regulated by ethylene. In contrast to the results of Whitehead and Vasiljevic, Woltering *et al.* (1993a) could not confirm an effect on flower life after application of octanoic and decanoic acids onto the stigma.

Blocking ethylene binding sites

The ethylene response is presumably mediated by binding of ethylene to a specific ethylene receptor whose activation signal is transduced via a (not fully identified) signal transduction pathway (Figure 1). An ethylene receptor has not yet been characterized. Recently, Hall *et al.* (1993) have purified two ethylene binding proteins, which may be part of the receptor complex. Biochemical characterization of these proteins may provide more insight into the process of ethylene binding, perception and signal transduction. Several compounds have been identified that can interfere with the ethylene receptor complex, leading to reduced ethylene binding (reviewed by Sisler, 1991). The most important compounds will be described in the next paragraph.

The application of silver ions to the flowers substantially reduced ethylene binding. Silver is supplied as an ionic complex with sodium thiosulphate (STS), which is more mobile in plant tissue and less phytotoxic. Carnation flowers treated with STS did not exhibit the climacteric rise in ethylene production nor did they accumulate ACC. This resulted in an extended vase life (Veen, 1986). In commercial practice STS is used to improve cut flower quality in carnations. The potential environmental concerns about pretreatment of flowers with STS, a heavy metal-containing compound, urged the need for developing a substitute means of controlling flower senescence.

Like STS, application of NBD also reduces ethylene binding activity. Petals in the climacteric exhibit a rapid decline in ethylene biosynthesis and ACC synthase activity when treated with NBD (Peiser, 1986; Wang and Woodson, 1989; Woodson *et al.*, 1992). NBD, being volatile, is a useful experimental tool. Its foul odour and toxicity to humans, however, make it of no practical use.

Diazocyclopentadiene (DACP) is another effective inhibitor of ethylene responses and senescence in cut carnations. After exposure to light, the binding sites in carnations preincubated with DACP are inactivated, leading to inhibition of the ethylene response. It was suggested that the toxic DACP is as effective as STS for preserving carnations (Sisler *et al.*, 1993).

Ethylene signal transduction

The ethylene signal transduction pathway has been analyzed both in a biochemical and in a genetic way. Biochemical experiments have provided evidence that calcium is required for ethylene signal transduction (Raz and Fluhr, 1992). The use of specific kinase and phosphatase inhibitors showed that phosphorylation events are essential for the ethylene-mediated pathogenesis response in tobacco plants (Raz and Fluhr, 1993).

The so-called 'triple response' to ethylene of etiolated *Arabidopsis* seedlings provides an elegant way to identify mutants with genetic defects in ethylene signal transduction. Etiolated wild type seedlings that are exposed to ethylene exhibit three distinct morphological changes: (1) inhibition of stem and root elongation, (2) radial stem expansion, and (3) loss of geotropism. The screening for triple response mutants resulted in the isolation of *Arabidopsis* plants which failed to respond to ethylene and mutants that constitutively displayed this response. All identified mutants have been reviewed recently by Kieber and Ecker (1993). At least seven different loci are involved in ethylene signal transduction. Three classes of ethylene-response mutants have been identified: (1) mutants that are insensitive to ethylene, (2) mutants that display ethylene responses in the absence of the hormone, and (3) mutants that are affected only in specific tissues. One of the well-characterized ethylene-resistant *Arabidopsis* mutants is *etr1* (Bleecker *et al.*, 1988). Four dominant mutant alleles of *etr1* have been identified. The ethylene binding was reduced 82 %, suggesting that the Etr1 protein is either the ethylene receptor or a protein that influences the function of the ethylene receptor. Analysis of the *etr1* mutant revealed the absence of all tested ethylene responses in various parts of the plant. This suggests that the Etr1 protein acts early in the ethylene signal transduction pathway. The corresponding *etr1* gene was isolated by chromosome walking (Chang *et al.*, 1993). Although the sequence of the amino-terminal half of the deduced Etr1 protein appears to be novel, the carboxy-terminal half shares homology with both proteins (the sensor and the regulator) of the bacterial two-component systems. In these systems a signal

perceived by the sensor is transduced via phosphorylation to the regulator, which in turn induces a response. This suggests that an early step in ethylene signal transduction in plants may involve transfer of phosphate.

The recessive *ctr1 Arabidopsis* mutant constitutively exhibits phenotypes observed in plants treated with ethylene, suggesting that the Ctr1 protein is a negative regulator of the ethylene response. Indeed, *ctr1* plants show constitutive expression of ethylene-regulated genes. Kieber *et al.* (1993) have cloned the *ctr1* gene. They demonstrated that *ctr1* was positioned downstream from the *etr1* gene in the ethylene signal transduction pathway. The gene encodes a putative serine/threonine protein kinase that is most closely related to the Raf protein kinase family. The identification of intermediates in the ethylene signal-transduction pathway is critical to our understanding of ethylene action.

Ethylene-resistant mutants in carnation

Ethylene-resistant mutants in carnation can teach us more about the process of flower senescence, because they represent genetic differences in the capacity to synthesize and respond to ethylene (Brandt and Woodson, 1992). Some examples of mutants with an extended vase-life are summarized in this section.

The mutant 'Killer' did not show a climacteric ethylene response. Besides the polyamine metabolism differed from other carnation cultivars (Serrano *et al.*, 1991). The sensitivity to ethylene of 'Sandrosa' flowers diminished with age, whereas other carnation cultivars showed increased ethylene sensitivity with age. During senescence of 'Sandrosa' flowers neither in-rolling symptoms nor a rise in the rate of ethylene production was observed. A transient rise in the activity of ACC oxidase occurred, but the activity of ACC synthase remained low (Mayak and Tirosh, 1993).

The cultivars 'Sandra' and 'Chinera' were found to have vase lives about twice that of 'White Sim' (Wu *et al.*, 1991a, 1991b). In all cases the long vase life of these cultivars is associated with the absence, reduction or delay of ethylene production. In 'Sandra', the capacity to synthesize ACC is low. Treatment with ethylene induced rapid wilting, suggesting that the ethylene response pathway was not affected. Woltering *et al.* (1993b) compared the vase life of the cultivar 'Chinera' and its progeny 'Epomeo' to 'White Sim'. Both in 'Chinera' and 'Epomeo', the increase in ACC synthase, ACC oxidase, ethylene production and membrane permeability, associated with the start of the senescence process, occurred later, and the maximum levels during senescence were much lower than in 'White Sim'. Ethylene dose-response experiments showed lower

ethylene sensitivity in 'Chinera' and 'Epomeo'. Wu *et al.* (1991b) used an isotope competition technique to show that the number of binding sites for ethylene was similar in 'Chinera' and 'White Sim'. Binding affinity was less in 'Chinera' and this may be the basis for its lower sensitivity.

Clearly, genetic mutations affecting ethylene synthesis or responsiveness are valuable tools in the study of flower senescence. Woltering *et al.* (1993b) showed that reduced ethylene sensitivity is inheritable. Improvement of carnation vase-life by conventional breeding programs using 'Chinera' or 'Epomeo' seems therefore feasible. Minor changes in the capacity to respond to ethylene may have commercially interesting effects on the vase life.

5 Senescence-related gene expression

The ethylene response finally leads to the synthesis of proteins involved in senescence (Woodson, 1987). The ethylene response is, at least partly, mediated via changes in the level of mRNAs (Woodson and Lawton, 1988; Lawton *et al.*, 1989). By means of differential screening approaches, several senescence related cDNAs were isolated and characterized (Woodson and Lawton, 1988; Lawton *et al.*, 1989). Some of these genes play a direct role in the biochemical events that occur during senescence, such as the gene encoding glutathione S-transferase (GST) (Meyer *et al.*, 1991) and the pSR132 gene. Comparison of the predicted amino acid sequence of pSR132 with other proteins revealed significant homology with carboxyphosphonoenolpyruvate mutase from *Streptomyces hygroscopicus* and phosphoenolpyruvate mutase from *Tetrahymena pyriformis*. These enzymes are involved in the formation of C-P bonds in the biosynthesis of phosphonates. It was suggested that the pSR132 polypeptide is involved in membrane turnover during senescence (Wang *et al.*, 1993).

The differential screening approaches also led to the isolation of the genes encoding ACC synthase and ACC oxidase, which are autocatalytically induced during the ethylene climacteric. Treatment of preclimacteric flowers with ethylene led to the accumulation of these senescence-related mRNAs within three hours. In carnation petals treated with STS prior to the addition of ethylene, no effect could be observed. This indicates that ethylene action is required for this mRNA accumulation.

The senescence-related mRNAs isolated so far can be divided into two classes

(Lawton *et al.*, 1990). One class of mRNAs accumulated mainly in petals during the ethylene climacteric or in response to ethylene addition. Accumulation of these mRNAs required the presence of ethylene, since their level decreased upon addition of the ethylene response inhibitor STS or the ethylene synthesis inhibitor AOA. The second class is represented by the pSR5 cDNA clone. This mRNA accumulated prior to the ethylene climacteric in petals and leaves. During the climacteric, its level remained high and was not influenced by treatment with inhibitors of ethylene synthesis or action. This suggests that expression of this gene is not exclusively dependent on ethylene. Nevertheless, in preclimacteric petals, the pSR5 transcript did accumulate within 0.5 h after ethylene treatment. The sequence and function of the pSR5 gene is not known yet. It is clear that regulation of gene expression by ethylene is organ specific for one gene, while for another it is not. In addition, each gene displays a unique ethylene dose-response curve.

In tomato, the ethylene-dependent mRNA accumulation of the E8 gene was mainly mediated via transcription initiation rather than via increased mRNA stability, since the 5′ promoter region was sufficient to confer ethylene responsiveness to a downstream-fused reporter gene (Deikman and Fischer, 1988; Deikman *et al.*, 1992). Also in carnation, it was shown that the promoter region of the senescence-related gene pSR8 is sufficient to confer ethylene-responsiveness upon a chimeric reporter gene (Itzhaki and Woodson, 1993). Analysis of the products of ethylene-induced genes and examination of the control of their expression should provide important information about the way in which ethylene controls senescence.

Gel-retardation and footprinting experiments with the ethylene-responsive E4 fruit-ripening gene from tomato revealed a specific ethylene-dependent interaction of a DNA binding protein with *cis*-sequences in the 5′ upstream region. This region was also found to be essential for ethylene-dependent gene expression (Montgomery *et al.*, 1993). Although there has been a lot of progress in the analysis of ethylene-dependent promoters, it is not possible to define a consensus sequence for ethylene-regulated promoters yet. In addition to transcriptional processes, also post-transcriptional processes are involved in the ethylene dependent gene expression (Lincoln and Fischer, 1988).

6 Improved postharvest longevity by genetic engineering

The use of molecular cloning techniques led to the isolation of genes involved in ethylene biosynthesis and action. This provides a means to construct longevity mutants in carnation by genetic engineering. Several strategies have been followed, as discussed below.

Inhibition of senescence related gene expression

The senescence process can be delayed by the selective down regulation of senescence-related genes. This can be accomplished by expressing a copy of the target gene in the inverse orientation, resulting in the formation of an antisense RNA transcript. Antisense RNA is thought to induce a dramatic decrease in the level of its complementary mRNA by the formation of a double stranded RNA intermediate (Kooter and Mol, 1993). In the next paragraphs several examples will be described.

Antisense ACC oxidase - Introduction of an antisense pTOM13 gene into tomato plants led to a dramatic decrease in ethylene production in ripening fruit. The endogenous pTOM13 mRNA level was reduced to 0.5 % of its normal wild-type level. The fruits had a strongly reduced capacity to convert ACC to ethylene, which provided the first evidence that pTOM13 encoded the ACC oxidase enzyme (Hamilton *et al.*, 1990). Ripening of these fruits was greatly delayed unless they were treated with ethylene. This led to reversal of the antisense phenotype and resulted in fruits that were indistinguishable from naturally ripened fruits (Picton *et al.*, 1993).

The development of methods for regeneration and transformation of carnation opens the way for genetic modification of carnation (Lu *et al.*, 1991, Van Altvorst *et al.*, 1992; Van Altvorst, 1994). Recently, the expression of the carnation ACC oxidase gene has been suppressed with an antisense RNA approach (Michael *et al.*, 1993). Analysis of the mutant revealed that ethylene production was reduced by 90 % when peak synthesis would be expected. The flowers of this mutant showed inhibition of petal-inrolling and an increased post-harvest life. This mutant, however, also contained less petals than its parent and these petals showed a slight decrease in pigmentation. Further analysis of other plants should provide evidence whether these pleiotropic effects are due to the antisense inhibition or to somaclonal variation.

Antisense ACC synthase - Oeller *et al.* (1991) used antisense ACC synthase constructs to inhibit fruit ripening in tomato. During tomato fruit ripening, two ACC synthase genes are expressed. Expression of antisense RNA from one gene resulted in an almost complete inhibition of mRNA accumulation of both ripening-induced ACC synthase genes (Oeller *et al.*, 1991) and a severe inhibition of ethylene production (99.5 % inhibition). Antisense fruits failed to ripen, they never turned red and soft or developed an aroma. The dramatic inhibition of ethylene production can be attributed to the short half life of the ACC synthase enzyme (Kende and Boller, 1981; Acaster and Kende, 1983).

Antisense E8 - When an antisense construct of the ethylene regulated tomato E8 gene was expressed in tomato plants, ethylene production during fruit ripening was up to six-fold enhanced (Penarrubia *et al.*, 1992). This indicates that E8 has a negative effect on ethylene production during fruit ripening. The function of the E8 gene is still unknown.

ACC conversion

Since ACC is known to participate only in the ethylene synthesis pathway, conversion of ACC may result in inhibition of ethylene synthesis. This can be achieved in several ways.

ACC deaminase - ACC levels can be modulated by the expression of the *Pseudomonas* ACC deaminase gene in plants. The ACC deaminase enzyme metabolizes ACC to α-ketobutyrate. To inhibit ethylene production, Klee *et al.* (1991) overexpressed the ACC deaminase gene in transgenic tomato plants. This approach led to 90-97 % inhibition of ethylene production during fruit ripening. Fruits from these plants showed significant delays in ripening and they remained firm for at least 6 weeks longer than nontransgenic control fruits. Reduction in ethylene synthesis in transgenic plants did not cause any apparent vegetative phenotypic abnormalities.

ACC malonyl-transferase - The ACC content in tissue may also be modified by influencing the enzyme ACC malonyl transferase, which catalyzes the conversion of ACC to MACC. ACC-malonylation is known to participate in the regulation of ethylene production (Amrhein *et al.*, 1981). ACC malonyl-transferase has been partially purified from mung bean hypocotyls (Kionka and Amhrein, 1984). Genes encoding ACC malonyl-transferase have not been

isolated to date.

Modification of ethylene 'sensitivity'

Carnation petals are extremely sensitive to exogenous ethylene. Genetic modification of the ethylene response could therefore be an advantage over modification of ethylene synthesis. For example, introduction of the dominant *Arabidopsis etr1-1* gene in wild type *Arabidopsis* plants resulted in plants that were completely insensitive to ethylene (Chang *et al.*, 1993). In analogy with *Arabidopsis*, the introduction of a dominant gene like *etr1-1*, might be an attractive way to confer complete ethylene insensitivity in carnation.

7 Concluding remarks

It is clear that the developmentally regulated increase in ethylene production and ethylene sensitivity in ageing flower petals is responsible for the induction of many biochemical processes that lead to programmed organ death. Ethylene is the regulatory molecule for flower senescence and is continuously required for the control of the senescence processes once initiated by ethylene. Ethylene is autocatalytically produced. The increase in ethylene biosynthesis during petal senescence of carnation is the result of a coordinate increase in the expression of ACC synthase and ACC oxidase genes. Molecular genetic and biochemical studies are beginning to unravel the process that control ethylene biosynthesis, perception and signal transduction.

Based on present day knowledge, there are several ways to modify endogenous ethylene production. In tomato, reduction of ACC synthase or ACC oxidase levels by means of antisense genes has been shown to increase shelf life. In *Arabidopsis*, introduction of the *etr1-1* gene resulted in ethylene-insensitive plants. Other ways to control the longevity of carnation flowers might be the overexpression of metabolizing enzymes such as ACC deaminase or modification of the ethylene receptor. However, it is not clear yet whether these modified plants can be propagated without any harmful effect on their normal physiology, since ethylene is involved in many aspects of plant growth and development. Therefore, it may be necessary to confine the expression of the ethylene-modulating genes to developing flowers.

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Summary

The conventional method for the introduction of new traits in plants, known as cross breeding, is a random process in which two complete genomes are mixed. By subsequent selection of the progeny new cultivars can be generated with economically important traits. The possibility of altering genetic information by conventional breeding is however limited by the existence of crossing barriers. Genetic modification of plants is a technique that enables the transfer and incorporation of one or a few genes into the plant genome in a precise manner. The most important advantage of genetic modification is the fact that it is not hampered by crossing barriers. It therefore opens the possibility of introducing genes, even from outside the plant kingdom. Genetic modification requires precise knowledge of the gene involved, and as this knowledge is still very limited, only a few suitable genes are yet available. At the start of this research project, genetic modification of carnation was not possible. The aim of the research described in this thesis was to develop suitable methods for the regeneration and transformation of carnation. The research was carried out at Centre for Plant Breeding and Reproduction Research (CPRO-DLO) and was supported financially by five Dutch breeding companies and the Dutch Ministry of Economic Affairs. In chapter 1 an overview is given of the existing carnation cultivation and breeding problems. Present breeding objectives include diversification (development of new flower forms and colours), resistance to insects and diseases, increase of the quality and postharvest longevity. The use of genetic engineering in carnation breeding is also discussed in chapter 1.

At the beginning of this research project, shoot regeneration of carnation was only possible from petal explants. However, regenerants derived from petal explants were mostly hyperhydric and flowered prematurely. Leaf explants were therefore used as starting material in the regeneration experiments. It was possible to generate adventitious shoots at the transition region from stem to leaf: the leaf base. The position of leaves on the plant appeared to be important for subsequent regeneration. The youngest leaves (leaf position 1) showed the highest regeneration percentage. The shoot regeneration procedure was efficient, 65 % of the explants showed regeneration with an average of 10 shoots per explant. Regenerants from leaf explants did not show aberrant plant growth (Chapter 2).

A node of a carnation cutting bears two leaves. These two leaves of a pair responded differentially to regeneration. The leaves that were removed first showed a regeneration response of 5 % whereas leaves removed secondly showed a regeneration response of 66 %. This was explained by the fact that the leaf removed from the stem secondly retained more tissue from the leaf base-stem transition region. By making two incisions through the fused leaf bases prior to their removal, explants with equal amounts of adhering stem tissue could be obtained which resulted in a higher regeneration response (Chapter 3).
In addition to leaf explants, it was possible to generate adventitious shoots from stem and axillary bud explants. The shoot regeneration procedures were applicable for a wide range of cultivars and regeneration percentages were high for all explant types. Using axillary bud explants, the regeneration response was independent of the size of the bud and of its original position in the plant. In contrast, shoot regeneration from stem and leaf explants was dependent on their original position in the plant, with the youngest explants showing the highest level of regeneration. In stem explants, shoot regeneration originated from cells located at the wound surfaces of nodes (Chapter 4).

Transformation of carnation was carried out using *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens*. These soil bacteria are able to transfer DNA to plants, thereby causing tumor or root formation. Using the ability of *Agrobacterium* to introduce DNA into plants, suitable genes can be transfered to plant cells. The *Agrobacterium* strains used in the research project were able to transfer the kanamycin resistance gene. Transformed cells could grow on kanamycin containing medium, while non-transfromed cells died. Shortly after infection, the number of transformation events in a leaf explant was estimated using a reporter gene, the *gus* gene. Using a histochemical assay, the presence of the introduced *gus* gene in a cell was visualized as a blue spot.
Of all tested *Agrobacterium* strains, the wild type *A.rhizogenes* strain LBA9402 was the most efficient in transferring DNA to carnation cells. The transformation effiency was dependent on the age of the leaf explant, with the youngest leaves showing the largest number of explants with GUS-positive spots. Four weeks after infection, kanamycin resistant callus was obtained from the leaf explants. After six months, this callus was still GUS-positive. However, regeneration from this transgenic callus was not possible (Chapter 5).
The susceptibility of carnation leaf explants to *A.tumefaciens* infection was

tested using 20 cultivars. Based on the regeneration and transformation response, five cultivars were selected for subsequent experiments. Four weeks after transformation, the first GUS-positive shoot primordia were visible. Despite selection on kanamycin-containing medium, only a small fraction (1 - 3%) of the adventitious shoots was transgenic. Apparently, regeneration was not fully inhibited by kanamycin. The trangenic plants were GUS-positive and able to root on kanamycin. Control plants however failed to root under these conditions. A total of nineteen transgenic plants was generated from five cultivars (one spray and four Sim type carnations). Seventeen of the nineteen transformants were obtained from the youngest leaves (leaf position 1). The two other plants were generated from leaf position 2 (the second youngest leaf). The transformation percentage (the number of transgenic shoots per 100 leaves) varied from 0.1 - 0.4 % after infection with the *A.tumefaciens* strain AGLO(p35SGUSint) and from 1 - 1.5 % after infection with AGLO(pCGN7001). The number of introduced gene copies varied between the transgenic carnation plants from 1 to 7. By sexual crossing, it was shown that the introduced genes were inherited in a Mendelian fashion (Chapter 6).
Transgenic carnation plants were also obtained after infection of petal explants with AGLO(p35SGUSint) or AGLO(pCGN7001). However, only a small percentage (1 - 5 %) of the adventitious shoots was transgenic. In total, four transgenic plants were obtained. Southern analysis revealed that the transgenic plants contained the *nptII* gene. As described for adventitious shoot formation from petal explants, regenerants in transformation experiments were hyperhydric and flowered prematurely (Appendix of Chapter 6).

This research has resulted in more efficient methods for the formation of adventitious shoots in carnation and a procedure for *Agrobacterium*-mediated transformation. In continuation of the research project, the developed leaf transformation method will be optimized and used for the introduction of new traits. The longevity of cut carnation flowers will be improved by the modification of senescence-related genes. During flower senescence, the volatile hormone ethylene functions as the central regulator. The senescence of carnation flowers is associated with a climacteric increase in the production of ethylene. Carnation flowers are also sensitive to ethylene. Ethylene serves to initiate and to regulate the processes that finally lead to programmed organ death. The senescence of carnation flowers can be retarded by inhibitors of ethylene biosynthesis and inhibitors of the ethylene response. For a long time,

carnation growers have added chemical inhibitors (such as silver thiosulphate) to the vase-water. However, the environmental concerns about pretreatment of flowers with silver thiosulphate has stimulated the desire to develop an alternative means for controlling flower senescence. Using molecular techniques, the senescence process could possibly be delayed. In chapter 7, an overview is given of the (molecular) mechanisms underlying the regulation of ethylene biosynthesis and ethylene sensitivity. The potential of using genetic engineering techniques in order to extend the vase-life of carnation flowers is discussed.

Samenvatting

De klassieke methode om cultuurplanten aan te passen, de kruisingsveredeling, is een willekeurig proces waarbij twee complete sets erfelijke informatie worden gemengd. Door de nakomelingen te selecteren ontstaan nieuwe cultivars met economisch belangrijke eigenschappen. De mogelijkheden van het uitwisselen van genetische informatie door middel van kruisingen is gelimiteerd door het bestaan van kruisingsbarrières. Genetische modificatie van planten is een techniek die het mogelijk maakt om op een nauwkeurige manier één of enkele genen te introduceren in het erfelijk materiaal van een plant. Het belangrijkste voordeel van genetische modificatie t.o.v. kruisingsveredeling is dat er geen kruisingsbarrières bestaan, hetgeen de mogelijkheid geeft om soortvreemde eigenschappen te introduceren. Genetische modificatie vereist exacte kennis van het gen dat overgedragen moet worden. Aangezien dergelijke kennis nog steeds erg beperkt is, zijn er op het moment nog maar weinig genen beschikbaar. Bij aanvang van dit promotie-onderzoek was genetische modificatie in de anjerveredeling niet mogelijk. Het doel van het in dit proefschrift beschreven onderzoek was het ontwikkelen van geschikte methoden voor regeneratie en transformatie van anjer. Het onderzoeksproject werd uitgevoerd op het Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO) en werd mede gefinancierd door vijf Nederlandse anjerveredelings-bedrijven en het ministerie van Economische Zaken. In hoofdstuk 1 wordt een overzicht gegeven van de problemen die voorkomen in de anjerteelt en de anjerveredeling. De veredelings-speerpunten omvatten verbreding van het sortiment (nieuwe kleuren en vormen), resistentie tegen ziekten en plagen en verbetering van kwaliteit en houdbaarheid. In hoofdstuk 1 worden vervolgens de mogelijkheden besproken die biotechnologie in de anjerveredeling kan bieden.

Bij de start van het onderzoek was regeneratie, d.w.z. de vorming van adventieve scheuten, bij anjer alleen mogelijk uit explantaten van kroonbladeren. Regeneranten afkomstig van kroonbladeren waren meestal gevitrificeerd (glazig) en bloeiden te vroeg. Het bleek ook mogelijk te zijn om adventieve scheuten te induceren op bladexplantaten. De scheutregeneratie trad op in het overgangsgebied van stengel naar blad: de bladbasis. De positie van de bladeren in de plant was belangrijk. De hoogste regeneratie-percentages werden gevonden in de jongste bladeren (bladpositie 1). De procedure was efficiënt, 65

% van de bladexplantaten vertoonde regeneratie met gemiddeld 10 scheuten per explantaat. De regeneranten vertoonden geen afwijkende groei (Hoofdstuk 2).
Een knoop van een anjerstek heeft twee bladeren. Deze bladeren gaven in het onderzoek een verschillende regeneratie-respons. Van het eerste blad dat van de stengel gescheurd werd gaf gemiddeld 5 % regeneratie terwijl het tweede 66 % regeneratie gaf. Dit verschil werd veroorzaakt door de grotere hoeveelheid stengelweefsel, die bij het scheuren van het tweede blad werd meegenomen. Door eerst de zijkanten van een bladpaar in te snijden werden twee explantaten verkregen waarbij een evengroot deel van het stengelweefsel werd meegescheurd. Op deze wijze werd een hoger regeneratiepercentage verkregen (Hoofdstuk 3).
Behalve uit bladexplantaten bleek adventieve scheutvorming ook mogelijk uit stengel- en axillaire knopexplantaten. Regeneratie-percentages waren hoog voor alle drie de explantaat-types en de methoden werden met succes toegepast bij een groot aantal cultivars. De regeneratie uit axillaire knoppen was niet afhankelijk van de lengte van het geïsoleerde meristeem en van de positie van de knop aan de plant. Daarentegen werd de regeneratie van blad- en stengelexplantaten wel beïnvloed door de oorspronkelijke positie van het explantaat aan de plant: de jongste explantaten gaven de hoogste regeneratiepercentages. In stengelexplantaten vond regeneratie voornamelijk plaats op het wondvlak van een knoop (Hoofdstuk 4).

Transformatie van anjer werd uitgevoerd met behulp van *Agrobacterium tumefaciens* en *Agrobacterium rhizogenes*. Deze bodembacteriën zijn in staat DNA naar planten over te dragen en veroorzaken hierbij tumorvorming resp. overdadige wortelgroei. Van het vermogen van *Agrobacterium* om DNA over te dragen naar planten wordt gebruik gemaakt om gewenste genen in planten te introduceren. De *Agrobacterium* stammen die in het onderzoek gebruikt werden droegen het kanamycineresistentie-gen over. Getransformeerde plantencellen waren daardoor in staat te groeien op een medium met kanamycine, terwijl niet-getransformeerde cellen dood gingen. Het aantal transformatie gebeurtenissen in een stukje plantenweefsel kon snel en reeds kort na de infectie bepaald worden door gebruik te maken van een zogenaamd indicator gen, het *gus* gen. De aanwezigheid van het ingebrachte *gus* gen was te toetsen door een histochemische kleuring. Getransformeerde cellen waren na transformatie waar te nemen als blauwe spots.

Van alle getoetste stammen bleek de wild-type *A.rhizogenes* stam LBA9402 het meest efficiënt te zijn in de overdracht van DNA naar anjerblad. De transformatie-efficiëntie was afhankelijk van de leeftijd van het bladexplantaat; de jongste bladeren gaven de meeste bladexplantaten met GUS-positieve spots. Kanamycine-resistent callus werd na 4 weken van het explantaat geïsoleerd en na 6 maanden bleek het callus nog steeds GUS-positief te zijn. Regeneratie uit dit transgene callus was echter niet mogelijk (Hoofdstuk 5).

Vervolgens werd bij 20 cultivars de gevoeligheid van bladexplantaten voor *A.tumefaciens* getoetst. Op basis van regeneratie en transformatie werden vijf cultivars geselecteerd voor verder onderzoek. Vier weken na transformatie werden de eerste transgene scheutprimordia waargenomen. De adventieve scheuten werden geselecteerd op kanamycine-resistentie. Ondanks selectie op kanamycine-bevattend medium was slechts een klein deel (1-3 %) van de adventieve scheuten getransformeerd. De regeneratie werd dus niet volledig geremd door kanamycine. De selectiedruk werd verhoogd door elke keer bij het overzetten de blad-explantaten in kleinere stukken te snijden. Het percentage 'ontsnappers' (niet getransformeerde scheuten) werd op deze manier omlaag gebracht en nu was 20 % van de adventieve scheuten getransformeerd. De transgene planten waren GUS-positief en bewortelden op kanamycine. Controle planten bewortelden niet onder die condities. Er werden 19 onafhankelijke getransformeerde planten verkregen, afkomstig van vijf verschillende cultivars (vier Sim-typen en een trosanjer). Zeventien van de negentien transformanten waren afkomstig van explantaten van de jongste bladeren (positie 1). De andere twee waren van afkomstig van bladpositie 2 (op één na jongste blad). Het transformatie-percentage (het aantal transgene scheuten per 100 bladeren) varieerde na infectie met de bacterie-stam AGLO(p35SGUSint) van 0.1 tot 0.4 % en na infectie met AGLO(pCGN7001) van 1 - 1.5 %. Het aantal geïntroduceerde genen in de transgene anjerplanten varieerde van 1 tot 7. Door middel van kruisingen werd aangetoond dat de geïntroduceerde genen op een juiste wijze (mendeliaans) overerven (Hoofdstuk 6).

Transgene planten werden ook verkregen door infectie van kroonblad-explantaten met AGLO(p35SGUSint) of AGLO(pCGN7001). Slechts een klein percentage (1 - 5 %) van de adventieve scheuten was getransformeerd. In totaal zijn vier transgene planten verkregen. Met moleculair biologische technieken werd aangetoond dat de transformanten het *nptII* gen hadden. Zoals reeds beschreven voor adventieve scheuten uit kroonbladeren, hadden ook regeneranten uit de transformatie-experimenten veel last van vroege bloei en

vitrificatie (Appendix van hoofdstuk 6).

Het onderzoek heeft geleid tot efficiënte methoden voor vorming van adventieve scheuten in anjer en tot een methode voor transformatie. In toekomstig onderzoek zal de ontwikkelde transformatieprocedure verder geoptimaliseerd worden en vervolgens toegepast worden voor de introductie van nieuwe eigenschappen in anjer. In een vervolgproject zal gewerkt worden aan de verbetering van de houdbaarheid van anjerbloemen. Bij veroudering van anjerbloemen speelt het gasvormige hormoon ethyleen een belangrijke rol. Tijdens veroudering neemt de ethyleenproduktie in anjerbloemen versneld toe. Ethyleen start en regelt de processen die vervolgens leiden tot een gecontroleerde verwelking. De veroudering van anjerbloemen kan worden tegengegaan door de ethyleensynthese of de ethyleenrespons van de bloem te remmen. Anjerkwekers doen dit al jaren met succes door het toevoegen van chemische remmers (zoals zilverthiosulfaat) aan het vaaswater. Deze remmers zijn echter zeer milieu-onvriendelijk en duur. Een alternatief zou zijn om het ethyleen-metabolisme te beïnvloeden door gebruik te maken van moleculair biologische technieken. In hoofdstuk 7 wordt een overzicht gegeven van de literatuur die betrekking heeft op de regulatie van ethyleensynthese en de ethyleenrespons. Verschillende mogelijkheden voor het verbeteren van de houdbaarheid van anjer via genetische modificatie worden besproken.

Nawoord

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Het onderzoek is grotendeels op het lab uitgevoerd, maar voor het uitgangsmateriaal was toch de hulp van Bert van Haaren onmisbaar.

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Tijdens het schrijven van het proefschrift hebben vele collega's van het CPRO mijn manuscripten kritisch gelezen en van nuttige commentaar voorzien. Ook andere collega's van de afdeling Ontwikkelingsbiologie en mensen uit mijn directe werkomgeving op het CPRO zijn belangrijk geweest door alle hulp die zij mij verleend hebben en hun bijdrage aan de prettige werksfeer op het lab. Hierbij is een woord van dank op zijn plaats aan Jan de Jong, Hein van Holsteijn, Jan Custers en Hans Jansen.

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Anne-Claire

Curriculum vitae

Anne-Claire van Altvorst werd op 9 mei 1965 te Oss geboren. Na het behalen van het Atheneum diploma aan de Scholen Gemeenschap Dukenburg (SGD) te Nijmegen, werd in 1983 begonnen met de studie Plantenveredeling aan de Landbouw Universiteit Wageningen. Het examen werd in 1989 gehaald en bestond o.a. uit de afstudeervakken Celgenetica en Moleculaire Biologie (Prof. dr. ir. M. Koornneef en Dr. P. Zabel), een stage aan de Florida State University (Prof. G.W. Bates) en een stage bij MOGEN Int. (Dr. P.C. Sijmons en Dr. A. Hoekema).

Van maart 1989 tot maart 1990 werkte zij op de afdeling Biotechnologie bij het Instituut voor de Veredeling van Tuinbouwgewassen (IVT) te Wageningen. Anne-Claire was toen werkzaam aan het project 'Introduction of *rol* genes of *Agrobacterium rhizogenes* in tomato'.

Van maart 1990 tot maart 1993 werkte zij op het Centrum van Plantenveredelins- en Reproduktie Onderzoek (CPRO-DLO) binnen de afdeling Ontwikkelingsbiologie. Gedurende deze periode werd onder leiding van Dr. J.J.M. Dons het in dit proefschrift beschreven onderzoek verricht.

Sinds 1 maart 1993 is Anne-Claire van Altvorst in dienst van de Vereniging van Groothandelarijen in Bloemkwekerijprodukten (VGB) en zet zij haar werkzaamheden voort op de afdeling Ontwikkelingsbiologie van het CPRO-DLO.

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